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(54) Title: USE OF TRANSLATIONALLY ALTERED RNA TO CONFER RESISTANCE TO MAIZE DWARF MOSAIC VIRUS AND OTHER MONOCOTYLEDONOUS PLANT VIRUSES			
(57) Abstract <p>The present invention provides methods and compositions for inhibiting virus infection in susceptible monocotyledonous plants. The methods and compositions involve the production of translationally altered forms of messenger RNA sequence derived from the inhibited virus. The invention further provides structural and organizational information for the genome of strain B of maize dwarf mosaic virus. Methods for inhibiting MDMV-B infection are taught. These methods include the generation of transformed plants containing chimeric genes capable of expressing either MDMV-B proteins or translationally altered forms of messenger RNA sequences produced by MDMV-B.</p>			

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USE OF TRANSLATIONALLY ALTERED RNA TO CONFER RESISTANCE TO MAIZE
DWARF MOSAIC VIRUS AND OTHER MONOCOTYLEDONOUS PLANT VIRUSES

The invention relates generally to the genetic engineering of monocotyledonous plants to resist virus infection through the expression of inhibitory transcripts or proteins derived from the inhibited virus. In another aspect, the invention relates to the elucidation and characterization of the genomic structure and organization of a maize dwarf mosaic virus.

Plant viruses are a major problem in agriculture and cause significant losses in crop yield each year. In the past, available approaches for combating plant viruses were primarily limited to the selection of plant lines which exhibited genetic resistance to virus infection and the application of chemicals designed to protect plants from the organisms responsible for introducing the virus to the plant (i.e. viral vectors).

Recently, a number of approaches for combating plant viruses have been developed which are based upon the transformation of susceptible plant species with chimeric genes which express transcripts or proteins that inhibit viral infection. These approaches include genetically engineering plants to express viral coat protein or coat protein transcripts, viral replicases in unmodified or modified form, antisense genes or ribozymes targeting viral genomic RNA or transcripts, and altered viral transcripts (for a review, see Fitch, J.H. et al., *Ann. Rev. Microbiol.* 47: 739-763 (1993)). To apply any of these approaches, knowledge of the structure and organization of the genome of the target virus is necessary.

With respect to the expression of altered viral transcripts to confer viral resistance, limited success has been reported in dicotyledonous plants through the expression of viral coat protein transcripts which have been modified to render them incapable of translation. Expression of such "untranslatable" viral transcripts in tobacco has been reported to inhibit tobacco etch virus (Lindbo, J.A. et al., *Mol. Plant-Microbe Int.* 5(2): 144-153 (1992); Lindbo, J.A. et al., *Virology* 189: 725-733 (1992); WO 93/17098 to Dougherty, W.G. et al. (Sept. 2, 1993); Lindbo, J.A. et al., *The Plant Cell* 5: 1749-1759 (1993)), tomato spotted wilt virus (Pang, S. et al., *Bio/Technology* 11: 819-824 (1993); DeHaan et al., *Bio/Technology* 10: 1133-1137 (1992) and potato virus Y (Van der Vlugt R.A. et al., *Plant Mol. Biol.* 17: 431-439 (1991)).

The ability of such untranslatable RNAs to inhibit viral infection does not appear to be universal, however. Failure of such altered viral transcripts to inhibit viral infection have been reported for tobacco mosaic virus (Powell, P.A. et al., Virology 175: 124-130 (1990) and zucchini yellow mosaic virus (Fang, G. et al., Mol. Plant-Microbe Int. 6(3): 358-367 (1993), a potyvirus similar to tobacco etch virus. Additional unreported failures may also exist, since such negative results are rarely published.

The most prevalent virus infecting maize in the United States and Europe is maize dwarf mosaic virus (MDMV). This virus is classified as a member of a family of plant viruses known as the potyviruses. The potyviruses are the largest group of plant viruses and are characterized by a long, flexuous rod particle morphology and are non-persistently transmitted by aphid vectors (see Hollings, M. and Brunt, A., pages 732-807 of "Handbook of Plant Virus Infection and Comparative Diagnosis", ed. by E. Kurstak, pub. by Elsevier/North Holland Biomedical Press, Amsterdam (1981)). The potyviruses have a genome composed of a single strand positive sense messenger RNA molecule which is transcribed and translated as one polyprotein that is subsequently cleaved into its component parts. The family is composed of many taxonomic strains, with the two most common being strains A and B. These strains are differentiated by the ability of MDMV-A to infect johnsongrass which is the overwintering host. MDMV-A is primarily localized to the southeastern United States due to the occurrence of johnsongrass in this area. MDMV-B is more widespread and can be found in the U.S. corn belt and throughout Europe (i.e. Spain, France, and Italy). MDMV-B is the most economically important maize virus due to its widespread occurrence.

Viral diseases of maize result in an estimated 5% annual yield reduction as well as reduce overall plant health which results in increased susceptibility to other pathogens. Experimental trials using common maize inbreds and hybrids have shown yield reductions from MDMV as great as 35% in inoculated plots. MDMV is a major crop pest in maize where it causes mosaic symptoms and dwarfing of infecting plants, ultimately reducing crop yields (Knoke, J.K. et al., pages 235-281 of "Diseases of Cereals & Pulses", volume I, ed. by Singh, U.S. et al., pub. by Prentice Hall, Englewood Cliffs, NJ (1992)). When found in combination with maize chlorotic mottle virus (MCMV), a synergistic condition known as corn lethal necrosis results causing even more severe crop damage (see Uyemoto, J.K., pages 141-143 of "Proc. Int'l. Maize Virus Disease Colloq. & Workshop", ed. by Gordon, D.T. et al., pub. by Ohio State Univ. and Ohio Agric. Res. Dev. Center, Wooster, MA (1983).

The economic impact of yield losses due to MDMV has generated considerable interest in developing strategies to combat this virus. To date, however, only limited success has been achieved in reducing the adverse impact of this virus. Thus there remains a need to identify additional effective means for protecting host plants from MDMV.

Both strains A and B of MDMV are transmitted in nature by aphids in a non-persistent manner, thus insect control is not a practical control method. The most effective method of control of these diseases is the use of resistant germplasm. In maize, sources of resistant germplasm exist to both strains of the MDMV, but the efficacy of the resistance is somewhat variable and identification of this material can be difficult. Studies have shown that resistance to MDMV is not the result of a single, dominant gene, but rather being multigenic (2-5 genes). There has been an abundance of research on the development of alternative strategies for conferring resistance in transgenic plants. Most of these strategies have focused on the expression of viral genes (i.e. the viral coat protein) in plants as a means of conferring resistance. The benefits of these strategies are that the resistance can be developed to viruses in which effective natural resistance can not be identified and the resistance is more easily transferred to agronomically desirable plant lines. The majority of this work has focused on coat protein mediated resistance which is based on the expression of the viral coat protein in the plant. Coat protein mediated resistance has been particularly effective for some viruses (e.g. tobacco mosaic virus) but inconsistent for other viruses (e.g. potyviruses) when tested in model systems such as tobacco and in economically important grain crops such as maize, wheat, and rice.

More recently, another virus resistance strategy has been developed which conferred an immune phenotype in plants transformed with segments of virus sequence. The phenomenon has been termed RNA-mediated resistance and is thought to be similar to sense suppression or co-suppression described in other plant systems. Specifically, plants were transformed with a sequence encoding the virus coat protein which had been modified to cause premature termination during translation. The expression of this untranslatable viral coat protein sequence at high levels was hypothesized to activate a RNA degradation cycle which eliminated the transgene mRNA in a sequence specific manner. The pathway was then believed to be capable of also eliminating an infecting virus which contains sequence highly homologous (>90%) to the transgene sequence. Since the original description of RNA-mediated resistance (see Lindbo, J.A. et al., *Mol. Plant-Microbe Int.* 5(2): 144-153 (1992) and DeHaan et al., *Bio/Technology* 10: 1133-1137 (1992)), there have been additional descriptions of this form of resistance. Furthermore, it has been shown that

prior work thought to be resistance due to expression of a viral protein is more likely to be RNA-mediated resistance. However, this strategy has not been effective for all viruses (see Powell, P.A. et al., *Virology* 175: 124-130 (1990) and Fang, G. et al., *Mol. Plant-Microbe Int.* 6(3): 358-367 (1993)). The examples of RNA-mediated resistance have been limited to model dicot hosts such as tobacco and potato. It is not known if this resistance will be effective in monocots nor what factors will be necessary for induction of this resistance.

The genomic structure and organization of MDMV has remained largely uncharacterized except for the elucidation of viral coat protein coding sequences (see Frenkel, M. J. et al. *J. Gen. Virol.* 72:237-242, (1991); see also Murray, L.E. et al., *Bio/Technology* 11: 1559-1564 (1993)). As a result, it is currently not possible to apply many of the more recent recombinant-DNA based approaches that have been used for combating plant viruses to MDMV. These approaches require a more extensive understanding of the structure and organization of the genome of the target virus than is currently available for MDMV.

In one aspect, the present invention provides a method for protecting a monocotyledonous plant from infection by a virus by producing in such a plant an RNA molecule whose sequence corresponds, at least in part, to a mRNA or the plus strand RNA produced by the virus. The RNA molecule produced according to the method of the invention is modified so that it cannot be translated completely as compared to the viral RNA to which it corresponds. Included within this aspect of the invention are chimeric genes designed to express such modified RNA molecules in monocotyledonous plants, as well as monocotyledonous plants containing such chimeric genes stably integrated into their genome. Such plants and their progeny are protected from infection by monocotyledonous viruses that produce messenger or plus-sense RNA which share sequence identity with the modified RNA molecule encoded and expressed by the stably integrated chimeric gene.

Another aspect of the invention is based upon structural and organizational information that has been elucidated for the genome of strain B of Maize Dwarf Mosaic Virus (MDMV-B) upstream of the coat protein gene. Included in this aspect of the invention are chimeric genes designed to express coding sequences for MDMV-B proteins including the coat protein (nucleotides 7308-8291 of SEQ ID No. 1), the RNA dependent RNA polymerase (RdRp) (nucleotides 5745-7307 of SEQ ID No. 1), proteinase (nucleotides 4452-5744 of SEQ ID No. 1), a 6K protein (nucleotides 4293-4451 of SEQ ID No. 1), cylindrical inclusion protein (CIP) (nucleotides 2376-4292 of SEQ ID No. 1), P3 proteinase

(nucleotides 1134-2375 of SEQ ID No. 1), and a portion of the helper component-P2 proteinase (HC-Pro) (nucleotides 3-1133 of SEQ ID No. 1). Methods for protecting plants from MDMV infection by transforming them with these chimeric genes are included within this aspect of the invention along with the resulting transgenic plants and their progeny.

The MDMV-B coding sequences may also be modified according to the first aspect of the present invention so that the RNA derived therefrom cannot be properly translated. The present invention includes chimeric genes designed to express such translationally altered MDMV-B RNAs in plants. Methods for protecting plants from MDMV infection by transforming them with these chimeric genes are included within this aspect of the invention along with the resulting transgenic plants and their progeny.

The following sequences according to the invention are disclosed in the sequence listing:

- SEQ ID No. 1: Sequence of the polycistronic messenger RNA of maize dwarf mosaic virus, strain B.
- SEQ ID NO. 2: Sequence of the polyprotein encoded by the polycistronic messenger RNA of maize dwarf mosaic virus, strain B.
- SEQ ID No. 3: First internal control alcohol dehydrogenase PCR primer used in analysis of T₀ plants as described in Example 4.
- SEQ ID No. 4: Second internal control alcohol dehydrogenase PCR primer used in analysis of T₀ plants as described in Example 4.
- SEQ ID No. 5: First PCR primer for the synthetic PAT gene used in analysis of T₀ plants as described in Example 4.
- SEQ ID No. 6: Second PCR primer for the synthetic PAT gene used in analysis of T₀ plants as described in Example 4.
- SEQ ID No. 7: First PCR primer for the Nla proteinase gene used in analysis of T₀ plants as described in Example 4.
- SEQ ID No. 8: Second PCR primer for the Nla proteinase gene used in analysis of T₀ plants as described in Example 4.

For purposes of describing the present invention, the term "translationally altered RNA" is used to refer to a modified form of a naturally occurring messenger RNA sequence which cannot be completely translated compared to the unmodified, naturally occurring form. A translationally altered RNA may be incapable of being translated at all or it may be

capable of being partially translated into an attenuated peptide corresponding to a portion of the peptide encoded by the naturally occurring messenger RNA sequence from which the translationally altered RNA is derived.

The coding sequence for a naturally occurring viral RNA sequence may be modified to encode a translationally altered RNA, for example, by removing its ATG initiation codon or by utilizing a portion which does not include the initiation codon. Other means for translationally altering a naturally occurring viral RNA molecule include introducing one or more premature stop codons and/or interrupting the reading frame.

The basis for the present invention is two-fold. The first basis for the present invention is the discovery that reduced susceptibility to infection by a virus may be conferred upon a monocotyledonous plant by producing in the plant a translationally altered RNA molecule corresponding in sequence to a plus-sense or messenger RNA molecule of the target virus. The second basis for the present invention is the elucidation and characterization by the inventors of the genomic structure and organization of strain B of maize dwarf mosaic virus (MDMV-B). These two bases are addressed consecutively below and are both represented by the examples demonstrating resistance to MDMV-B via expression of a translationally altered RNA in a transgenic maize plant.

The first aspect of the present invention is directed to a general method for reducing the susceptibility of a monocotyledonous plant to viral infection by producing in the plant a translationally altered RNA molecule corresponding to a messenger RNA sequence of the target virus. Viruses infecting monocotyledonous plants will be referred to as monocotyledonous viruses. A method is provided for protecting progeny of a monocotyledonous parent plant from viral infection by transforming said parent plant with a chimeric gene comprising a monocotyledonous plant promoter operably linked to a nucleotide sequence derived from the genomic sequence of a virus infecting monocotyledonous plants, wherein said nucleotide sequence contains a modification rendering a messenger RNA transcribed from said nucleotide sequence incapable of complete translation, and obtaining progeny plants. Alternatively, said progeny of a parent plant can be protected from viral infection by breeding the parent plant with a monocotyledonous plant having an inheritable trait of resistance to infection due to its expression of a chimeric gene comprising a monocotyledonous plant promoter operably

linked to a nucleotide sequence derived from the genomic sequence of a virus infecting monocotyledonous plants, wherein said nucleotide sequence contains a modification rendering a messenger RNA transcribed from said nucleotide sequence incapable of complete translation

The preferred approach for producing the translationally altered RNA molecule in a monocotyledonous plant is by introducing a chimeric gene designed to express this molecule into the genome of the plant. Such a chimeric gene will consist of a plant promoter operably linked to a nucleotide sequence derived from the genomic sequence of a virus infecting monocotyledonous plants, wherein said nucleotide sequence contains a modification rendering a messenger RNA transcribed from said nucleotide sequence incapable of complete translation.

The promoter component may be any monocotyledonous plant promoter that is any promoter which is capable of regulating or directing the expression of an operably linked gene in the targeted monocotyledonous plant. Such promoters are well known in the art. Preferably, a promoter which is capable of directing strong constitutive expression is used. Such promoters include, but are not limited to, the maize ubiquitin promoter described in Toki et al., *Plant Physiol.* 100: 1503-1507 (1992), the maize phosphoenolpyruvate carboxylase (PEPC) promoter as described in Hudspeth, R.L. et al., *Plant Molec. Biol.* 12: 579-589 (1989), and the CaMV 35S promoter as described in Kay et al., *Science* 236: 1299-1302 (1987).

The coding sequence component comprises a sequence which, when transcribed, produces a translationally altered RNA molecule corresponding to a target viral sequence. The target viral sequence is a messenger RNA (mRNA) molecule of the target virus, or a portion thereof. Since the target viral sequence is naturally translatable when a translation initiation codon is present, it is modified so as to render it translationally altered. For any given target viral sequence, the skilled artisan will be able to determine various modifications which could be made to render the resulting RNA molecule translationally altered.

Translation of an mRNA molecule in a plant cell generally requires the presence of an initiation AUG codon followed by an uninterrupted string of amino acid codons (known as the reading frame) ending with a translational stop codon, which may be either UAA, UAG or UGA. A DNA molecule encoding a translatable mRNA molecule may be modified to encode a translationally altered RNA, for instance, by either removing the initiation ATG

codon, interrupting the reading frame, adding premature stop codons, or by a combination of these modifications.

Introduction of one or more premature stop codons (encoded by DNA codons TAA, TAG or TGA) in a target viral sequence may be accomplished by adding or deleting nucleotides or by modifying existing nucleotides using standard techniques such as site directed mutagenesis or mutagenesis by PCR. Adding or deleting nucleotides may have the additional benefit of interrupting the reading frame, which also has the effect of translationally altering the RNA molecule. While the addition of a premature stop codon anywhere along the length of the target viral sequence will render it translationally altered as that term is used herein to describe the invention, it is preferable to introduce such stop codons near the 5' end of the target viral mRNA so that any attenuated peptides which may be produced via partial translation are 20 amino acids or less in length.

The reading frame of a target viral sequence may be interrupted by the addition or deletion of nucleotides in the DNA coding sequence. As with the addition of premature stop codons, it is preferable to interrupt the reading frame near the 5' end of the target viral RNA so that any attenuated peptides corresponding to a portion of the peptide encoded by the target viral RNA which may be produced via partial translation are 20 amino acids or less in length.

Another way to translationally alter the target viral sequence is to remove the translation initiation codon, which will be an ATG. This may be accomplished simply by choosing a target viral sequence which does not include the translation initiation codon. Alternatively, this may be accomplished by disrupting the ATG codon either by adding, deleting or modifying nucleotides within this codon using standard techniques.

Any messenger RNA molecule produced by the target monocotyledonous virus, or any portion of such a molecule, may be used as the target viral sequence. The target viral sequence is preferably at least 120 nucleotides in length, more preferably at least 250 nucleotides in length, and most preferably at least 500 nucleotides in length.

A translationally altered viral RNA according to the invention includes any modified form of a naturally occurring viral messenger RNA sequence which cannot be completely translated as compared to the unmodified, naturally occurring form. Thus a translationally altered viral RNA may either be incapable of being translated at all, or it may be capable of translating an attenuated peptide corresponding to a portion of the peptide encoded by the target viral sequence used as a template.

The inhibitory effect of a translationally altered viral RNA is contemplated to be based, at least in part, upon its effect on host cell degradation mechanisms. Production of a translationally altered viral RNA in a plant cell is contemplated to trigger one or more cellular RNA degradation mechanisms which target the translationally altered viral RNA, as well as any corresponding homologous unaltered viral RNA molecules which may be present in the cell (see, e.g. page 550 of Dougherty, W.G. et al., *Mol. Plant-Microbe Int.* 7(5): 544-552 (1994); Chasan, R., *The Plant Cell* 6: 1329-1331 (1994)).

The ability to translate an attenuated peptide, particularly a short peptide less than 20 amino acids, is contemplated to enhance the triggering effect of the translationally altered viral RNA upon host cell RNA degradation pathways contemplated to play a role in inhibition of viral infection. Thus translationally altered RNAs which are capable of translating an attenuated peptide are preferred. More preferably, the translationally altered viral RNA is capable of translating an attenuated peptide less than 20 amino acids in length. For target viral RNAs which do not include a translation initiation codon, one may be added in conjunction with the addition of a premature stop codon or interruption of the reading frame to create a translationally altered RNA capable of translating an attenuated peptide (see, for example, the construct pCIB5018 described in Example 4).

Target viral sequences may be selected from the group consisting of a potyvirus, luteovirus, tenuivirus, carmovirus, machlovirus, geminivirus and reovirus sequences and may correspond to the coding sequence for any viral protein, such as a viral coat protein, replicase, proteinase, inclusion body protein, helicase, 6K protein and VPg. Such sequences are well known for several monocotyledonous viruses including, but not limited to, MDMV (see SEQ ID NO. 1), Sugarcane mosaic virus (partial sequence; see Frenkel, M. J. et al. *J. Gen. Virol.* 72:237-242, (1991)), Johnsongrass mosaic virus (partial sequence) (see Gough, K. H. et al., *J. Gen. Virol.* 68:297-304, (1987)), maize chlorotic mottle virus (see Nutter, R. C. et al. *Nucleic Acids Research* 17:3163-3177, (1989)), maize chlorotic dwarf virus (see WO 94/21796), maize rough dwarf virus (partial sequence) (see Marzachi, C. et al. *Virology* 180:518-526, (1991)), maize stripe virus (partial sequence) (see Huilet, L. et al. *Virology* 182:47-53, (1991); Huilet, L. et al. *J. Gen. Virol.* 73:1603-1607, (1992); Huilet, L. et al. *GenBank Accession Number* L3446, (1993)), maize streak virus (see Mullineaux, P. M. et al. *EMBO J.* 3:3063-3068, (1984)), barley yellow dwarf virus (see Larkins, B. A. et al. *J. Gen. Virol.* 72:2347-2355, (1991)), and wheat spindle streak virus (partial sequence) (see Sohn, A. et al. *Arch. Virol.* 135:279-292, (1994)).

Suitable host plants which may benefit from the production of translationally altered viral RNA such as altered MDMV RNA include any monocotyledonous species which are susceptible to viral infection, particularly infection by a member of the potyvirus family. In particular, suitable host plants include maize, wheat, sugarcane and sorghum.

In a preferred embodiment, the target viral sequence used is a coding sequence which is identical or highly homologous among two or more monocotyledonous viruses or virus strains. Expression of a translationally altered RNA in a monocotyledonous plant based on such a shared sequence is contemplated to inhibit infection by any of the viruses which produce a messenger RNA having homology with the target viral sequence.

A second aspect of the present invention is based upon the elucidation and characterization by the inventors of the genomic structure and organization of strain B of maize dwarf mosaic virus (MDMV-B). Previously, only the genomic sequence of the MDMV-B coat protein was known (see Frenkel, M. J. et al., J. Gen. Virol. 72: 237-242 (1991)). As a result of the disclosed invention it is now possible to apply many of the more recent recombinant-DNA based approaches that have been used for combating plant viruses to MDMV such as the use of chimeric genes comprising a plant promoter operably linked to a nucleotide sequence derived from the genomic sequence of maize dwarf mosaic virus strain B encoding a viral protein other than a coat protein, wherein transgenic expression of said chimeric genes in a plant inhibits infection of said plant with said virus.

The MDMV-B positive strand RNA genome is believed to be approximately 10,000 bases in length based on the length of other potyviruses. The sequence of 8530 nucleotides beginning at the 3' end of the MDMV-B genome is set forth in SEQ ID NO: 1. A single long open reading frame was identified within this sequence of the viral genome and the polyprotein amino acid sequence encoded by this open reading frame is provided in SEQ ID NO: 2. With the sequence information provided, this viral genome can be isolated and cloned using a variety of standard genetic engineering techniques well known to those of skill in the art. Three DNA fragments covering 85% of the MDMV-B genome have been cloned into a Bluescript II SK plasmid backbone (Stratagene), transformed and propagated in the E. coli cell line HB101, and deposited on June 29, 1995 with the Midwest Area National Center for Agricultural Utilization Research (formerly known as the National Regional Research Lab and still referred to by the corresponding acronym "NRRL"). One of the plasmids designated "1-47" and deposited under the accession No. NRRL B-21479

contains nucleotides 3252-8530 of the MDMV-B genome. Another plasmid designated "2-24" and deposited under the accession No. NRRL B-21480 contains nucleotides 1866-3317 of the MDMV-B genome. Yet another plasmid designated "9-1-5" and deposited under the accession No. NRRL B-21481 contains nucleotides 1-2122 of the MDMV-B genome.

The polyprotein encoded by the MDMV-B genome includes a single coat protein designated CP whose coding sequence extends from nucleotide 7308 to 8291 of SEQ ID No. 1 and whose amino acid sequence extends from amino acid 2436 to 2763 of SEQ ID No. 2. The MDMV-B polyprotein is also contemplated to include a replicase protein, three proteinases, a 6K protein, a helper component, proteins involved in viral movement in the host plant (both cell to cell and long distance transport), a helicase protein and a VPg protein.

MDMV-B is contemplated to contain a serine-like proteinase analogous to serine-like proteinases that have been identified in related potyviruses. These serine-like proteinases have a characteristic catalytic domain of three amino acids consisting of a histidine at position 1 of the domain, an aspartic acid at the second position, and a cysteine at the third (see Bazan, J. F. and Fletterick, R. J., Proc. Natl. Acad. Sci. USA 85: 7872-7876 (1988)). These amino acids are separated in the primary amino acid sequence by a region spanning approximately 140 amino acids. The intervening sequences between each of the catalytic domain sequences exhibits additional limited homology among the known proteinases (see Bazan, J. F. and Fletterick, R. J., Proc. Natl. Acad. Sci. USA 85: 7872-7876 (1988)). Based upon comparison with the known proteinase sequences, the MDMV-B proteinase catalytic domain is contemplated to span a 105 amino acid sequence from position 1718 to 1823 of SEQ ID No: 2 with the three catalytic residues occurring at amino acids 1718, 1753, and 1823 of SEQ ID No. 2.

MDMV-B is also contemplated to contain a second proteinase analogous to the cysteine proteinases that have been identified in related potyviruses. The active-site residues form a catalytic diad made up of a conserved cysteine and histidine which are separated by 72 amino acids (see Oh, C. and Carrington, J. C., Virology 173:692-699, (1989)). This proteinase is located within the carboxy-terminus of the HC-Pro region of the potyvirus polyprotein. Based upon comparison with the known proteinase sequences of tobacco etch virus, the MDMV-B HC-Pro proteinase domain is contemplated to span a 74 amino acid region from position 263 to 336 of SEQ ID No: 2 with the two catalytic residues occurring at amino acids 263 and 336.

The location of the MDMV-B putative helicase domain can be identified based on the homology with other known viral helicase domains (see Gorbalenya, A. E. et al., *Nucleic Acids Research* 17 (12):4713-4730, (1989)). The helicase domain consists of seven distinct highly conserved segments which correspond to the NTP-binding motif. The primary consensus site consists of a glycine at position 1 of the motif, glycine at position 3, lysine at position 4, and either a serine or threonine at position 5 (see Gorbalenya, A. E. et al. *supra*). The conserved helicase domain is located in the MDMV-B genome within a region encoding the cylindrical inclusion protein (CIP) and is found from amino acids 880 to 1010 of SEQ ID No: 2. The conserved domain (GxGDS) is located at amino acids 883, 885, 886, and 887 of SEQ ID No: 2.

The coding sequence for the replicase gene of MDMV-B may also be determined by the location of conserved motifs common to viral replicase genes and by identification of putative viral proteinase cleavage sites bordering the replicase coding sequence. Conserved motifs have been found in other viral replicases. In particular, the conserved amino acid motif GDD (known as domain C) is the hallmark consensus sequence for all RNA- dependent replicases (Poch et al. *EMBO* 8: 3867-3874 (1989)). This conserved motif is found at amino acids 2266-2268 in the MDMV-B open reading frame (SEQ ID No: 2). Two additional conserved motifs characteristic of a plant viral replicase have been identified and designated as domain A and B (Poch et al., *supra*). Domain A is a 17 amino acid sequence with two centrally conserved amino acids which are present in the MDMV-B genome at amino acids 2163 and 2168 of SEQ ID No: 2. Domain B is a 10 amino acid sequence consisting of 5 conserved amino acids which are present in the MDMV-B genome at amino acids 2222, 2223, 2224, 2225 and 2226 of SEQ ID No: 2.

The isolated MDMV-B genomic sequences taught by the present invention are particularly useful for the development of viral resistance in susceptible host plants. With the information provided by the present invention, several approaches for inhibiting plant virus infection in susceptible plant hosts which involve expressing in such hosts various inhibitory transcripts or proteins derived from the target virus genome may now be applied to MDMV.

Use of translationally altered RNA in a method for producing a monocotyledonous plant with an inheritable trait of resistance to infection by a maize dwarf mosaic virus comprising transforming said plant with a chimeric gene comprising a monocotyledonous

plant promoter operably linked to a nucleotide sequence derived from the genomic sequence of a maize dwarf mosaic virus, wherein said nucleotide sequence contains a modification rendering a messenger RNA transcribed from said nucleotide sequence incapable of complete translation, may now be applied to MDMV-B, as demonstrated by Example 4.

Another approach which may be used to confer plant virus resistance is to express the gene of the target virus in the host plant (e.g. WO 94/18336 to Tumer et al. for potato leaf roll virus and WO 91/13542 to Zaitlin et al. for tobacco mosaic virus; herein incorporated by reference in their entirety). This approach may also be applied to MDMV-B using the information provided by the present invention.

For resistance strategies which depend upon expression of a viral replicase coding sequence in a transgenic plant, a cDNA clone encompassing nucleotides 5745 to 7307 of SEQ ID No: 1, contemplated to include the active domains of the MDMV-B can be used for plant transformation. More preferably, such strategies may be employed by transforming a plant with larger expressible fragments of the MDMV-B genome contemplated to encompass the entire replicase protein. In this case, the MDMV-B replicase would be cleaved from the encoded polypeptide when exposed to MDMV-B viral proteinase in the plant cell.

The MDMV-B replicase coding sequence may be engineered for recombinant expression in a monocotyledonous host plant which is normally susceptible to infection by MDMV-B. Expression of MDMV-B replicase in such a monocotyledonous host plant is contemplated to confer resistance to (i.e. inhibit) MDMV-B infection.

Suitable host plants which may benefit from application of any of the resistance approaches described above include any monocotyledonous species which are susceptible to infection by MDMV-B. In particular, suitable host plants are contemplated to include maize, sorghum and sugarcane.

To express inhibitory transcripts or proteins derived from the MDMV-B genome in a host plant cell, the corresponding coding sequence is operably linked to regulatory sequences which cause its expression in the chosen host plant cell. Examples of promoters capable of functioning in plants or plant cells, i.e., those capable of driving expression of the associated coding sequences such as MDMV-B CP in plant cells, include the cauliflower mosaic virus (CaMV) 19S or 35S promoters and CaMV double promoters; nopaline synthase promoters; pathogenesis-related (PR) protein promoters; small subunit of ribulose biphosphate carboxylase (ssuRUBISCO) promoters; plant ubiquitin gene

promoters; plant actin gene promoters; plant pith-preferred promoters, and the like. Preferred are the rice actin promoter (McElroy et al., Mol. Gen. Genet. 231: 150 (1991)), maize ubiquitin promoter (EP-A-342 926; Taylor et al., Plant Cell Rep. 12: 491 (1993); Toki et al., Plant Phys. 100:1503-1507 (1992)), a maize pith-preferred promoter (WO 93/07278 incorporated by reference herein in its entirety; in particular see Figure 24 and pages 27-28), and the Pr-1 promoter from tobacco, Arabidopsis, or maize (see EP-A-332 104). Also preferred are the 35S promoter and an enhanced or double 35S promoter such as that described in Kay et al., Science 236: 1299-1302 (1987) and the double 35S promoter cloned into pCGN2113, deposited as ATCC 40587. The promoters themselves may be modified to manipulate promoter strength to increase expression of MDMV-B coding sequences in accordance with art-recognized procedures.

The chimeric DNA construct(s) of the invention may contain multiple copies of a promoter or multiple copies of a particular coding sequence. In addition, the construct(s) may include coding sequences for markers and coding sequences for other peptides such as signal or transit peptides, each in proper reading frame with the other functional elements in the DNA molecule. The preparation of such constructs are within the ordinary level of skill in the art.

Since the MDMV-B proteins are naturally expressed as part of a polyprotein, each protein does not include its own translation initiation and translation stop codon. To express such proteins individually in the context of a chimeric gene, a translation initiation codon will need to be added immediately adjacent to the first codon if one does not occur as part of the coding sequence. The skilled artisan will recognize that addition of such a translation initiation codon will add a methionine amino acid to the end of the encoded protein. Such an addition is not contemplated to have any significant effect upon the properties of the protein. Also, a translation stop codon will need to be added to the chimeric gene immediately after the last codon of the protein if one does not naturally occur at this location.

Useful markers include peptides providing herbicide, antibiotic or drug resistance, such as, for example, resistance to hygromycin, kanamycin, G418, gentamycin, lincomycin, methotrexate, glyphosate, phosphinothricin, or the like. These markers can be used to select cells transformed with the chimeric DNA constructs of the invention from untransformed cells. Other useful markers are peptidic enzymes which can be easily detected by a visible reaction, for example a color reaction, for example luciferase, β -glucuronidase, or β -galactosidase.

Standard recombinant DNA and molecular cloning techniques used in the following examples are well known in the art and are described by J. Sambrook, E. F. Fritsch and T. Maniatis, Molecular Cloning: A Laboratory manual, Cold Spring Harbor laboratory, Cold Spring Harbor, NY (1989) and by T.J. Silhavy, M.L. Berman, and L.W. Enquist, Experiments with Gene Fusions, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (1984).

Example 1: Construction of Plant Transformation Vectors

Numerous transformation vectors are available for plant transformation, and the genes of this invention can be used in conjunction with any such vectors. The selection of vector for use will depend upon the preferred transformation technique and the target species for transformation. For certain target species, different antibiotic or herbicide selection markers may be preferred. Selection markers used routinely in transformation include the nptII gene which confers resistance to kanamycin and related antibiotics (Messing & Vierra, Gene 19: 259-268 (1982); Bevan et al., Nature 304:184-187 (1983)), the bar gene which confers resistance to the herbicide phosphinothricin (White et al., Nucl. Acids Res. 18: 1062 (1990), Spencer et al., Theor. Appl. Genet. 79: 625-631 (1990)), the hph gene which confers resistance to the antibiotic hygromycin (Blochinger & Diggelmann, Mol Cell Biol 4: 2929-2931 (1984)), and the dhfr gene, which confers resistance to methotrexate (Bourouis et al., EMBO J. 2(7): 1099-1104 (1983)).

1. Construction of Vectors Suitable for Agrobacterium Transformation

Many vectors are available for transformation using *Agrobacterium tumefaciens*. These typically carry at least one T-DNA border sequence and include vectors such as pBIN19 (Bevan, Nucl. Acids Res. (1984)). Below the construction of two typical vectors is described.

1.1. Construction of pCIB200 and pCIB2001

The binary vectors pCIB200 and pCIB2001 are used for the construction of recombinant vectors for use with *Agrobacterium* and was constructed in the following manner. pTJS75kan was created by NarI digestion of pTJS75 (Schmidhauser & Helinski, J Bacteriol. 164: 446-455 (1985)) allowing excision of the tetracycline-resistance gene,

followed by insertion of an *AccI* fragment from pUC4K carrying an NPTII gene (Messing & Vierra, *Gene* 19: 259-268 (1982); Bevan et al., *Nature* 304: 184-187 (1983); McBride et al., *Plant Molecular Biology* 14: 266-276 (1990)). *XhoI* linkers were ligated to the *EcoRV* fragment of pCIB7 which contains the left and right T-DNA borders, a plant selectable *nos/nptII* chimeric gene and the pUC polylinker (Rothstein et al., *Gene* 53: 153-161 (1987)), and the *XhoI*-digested fragment was cloned into *Sall*-digested pTJS75kan to create pCIB200 (see also EP-A-332 104, example 19). pCIB200 contains the following unique polylinker restriction sites: *EcoRI*, *SstI*, *KpnI*, *BglII*, *XbaI*, and *Sall*. pCIB2001 is a derivative of pCIB200 which was created by the insertion into the polylinker of additional restriction sites. Unique restriction sites in the polylinker of pCIB2001 are *EcoRI*, *SstI*, *KpnI*, *BglII*, *XbaI*, *Sall*, *MluI*, *BclI*, *AvrII*, *Apal*, *HpaI*, and *StuI*. pCIB2001, in addition to containing these unique restriction sites also has plant and bacterial kanamycin selection, left and right T-DNA borders for *Agrobacterium*-mediated transformation, the RK2-derived *trfA* function for mobilization between *E. coli* and other hosts, and the *OriT* and *OriV* functions also from RK2. The pCIB2001 polylinker is suitable for the cloning of plant expression cassettes containing their own regulatory signals.

1.2. Construction of pCIB10 and Hygromycin Selection Derivatives thereof

The binary vector pCIB10 contains a gene encoding kanamycin resistance for selection in plants, T-DNA right and left border sequences and incorporates sequences from the wide host-range plasmid pRK252 allowing it to replicate in both *E. coli* and *Agrobacterium*. Its construction is described by Rothstein et al., *Gene* 53: 153-161 (1987). Various derivatives of pCIB10 have been constructed which incorporate the gene for hygromycin B phosphotransferase described by Gritz et al., *Gene* 25: 179-188 (1983)). These derivatives enable selection of transgenic plant cells on hygromycin only (pCIB743), or hygromycin and kanamycin (pCIB715, pCIB717).

2. Construction of Vectors Suitable for non-*Agrobacterium* Transformation.

Transformation without the use of *Agrobacterium tumefaciens* circumvents the requirement for T-DNA sequences in the chosen transformation vector and consequently vectors lacking these sequences can be utilized in addition to vectors such as the ones described above which contain T-DNA sequences. Transformation techniques which do not rely on *Agrobacterium* include transformation via particle bombardment, protoplast uptake

(e.g. PEG and electroporation) and microinjection. The choice of vector depends largely on the preferred selection for the species being transformed. Below, the construction of some typical vectors is described.

2.1 Construction of pCIB3064

pCIB3064 is a pUC-derived vector suitable for direct gene transfer techniques in combination with selection by the herbicide basta (or phosphinothricin). The plasmid pCIB246 comprises the CaMV 35S promoter in operational fusion to the E. coli GUS gene and the CaMV 35S transcriptional terminator and is described in WO 93/07278. The 35S promoter of this vector contains two ATG sequences 5' of the start site. These sites were mutated using standard PCR techniques in such a way as to remove the ATGs and generate the restriction sites SspI and PvuII. The new restriction sites were 96 and 37 bp away from the unique Sall site and 101 and 42 bp away from the actual start site. The resultant derivative of pCIB246 was designated pCIB3025. The GUS gene was then excised from pCIB3025 by digestion with Sall and SacI, the termini rendered blunt and religated to generate plasmid pCIB3060. The plasmid pJIT82 was obtained from the John Innes Centre, Norwich and a 400 bp SmaI fragment containing the bar gene from *Streptomyces viridochromogenes* was excised and inserted into the HpaI site of pCIB3060 (Thompson et al. EMBO J 6: 2519-2523 (1987)). This generated pCIB3064 which comprises the bar gene under the control of the CaMV 35S promoter and terminator for herbicide selection, a gene for ampicillin resistance (for selection in E. coli) and a polylinker with the unique sites SphI, PstI, HindIII, and BamHI. This vector is suitable for the cloning of plant expression cassettes containing their own regulatory signals.

2.2 Construction of pSOG19 and pSOG35

pSOG35 is a transformation vector which utilizes the E. coli gene dihydrofolate reductase (DHFR) as a selectable marker conferring resistance to methotrexate. PCR was used to amplify the 35S promoter (~800 bp), intron 6 from the maize Adh1 gene (~550 bp) and 18 bp of the GUS untranslated leader sequence from pSOG10. A 250 bp fragment encoding the E. coli dihydrofolate reductase type II gene was also amplified by PCR and these two PCR fragments were assembled with a SacI-PstI fragment from pBI221 (Clontech) which comprised the pUC19 vector backbone and the nopaline synthase terminator. Assembly of these fragments generated pSOG19 which contains the 35S promoter in fusion with the intron 6 sequence, the GUS leader, the DHFR gene and the

nopaline synthase terminator. Replacement of the GUS leader in pSOG19 with the leader sequence from Maize Chlorotic Mottle Virus (MCMV) generated the vector pSOG35. pSOG19 and pSOG35 carry the pUC gene for ampicillin resistance and have HindIII, SphI, PstI and EcoRI sites available for the cloning of foreign sequences.

Example 2: Construction of Plant Expression Cassettes

Gene sequences intended for expression in transgenic plants are firstly assembled in expression cassettes behind a suitable promoter and upstream of a suitable transcription terminator to create a chimeric gene. These expression cassettes can then be easily transferred to the plant transformation vectors described above in Example 1.

Promoter Selection

The selection of a promoter used in expression cassettes or chimeric genes will determine the spatial and temporal expression pattern of the transgene in the transgenic plant. Selected promoters will express transgenes in specific cell types (such as leaf epidermal cells, mesophyll cells, root cortex cells) or in specific tissues or organs (roots, leaves or flowers, for example) and this selection will reflect the desired location of expression of the transgene. Alternatively, the selected promoter may drive expression of the gene under a light-induced or other temporally regulated promoter. A further alternative is that the selected promoter be chemically regulated. This would provide the possibility of inducing expression of the transgene only when desired and caused by treatment with a chemical inducer.

Transcriptional Terminators

A variety of transcriptional terminators are available for use in expression cassettes. These are responsible for the termination of transcription beyond the transgene and its correct polyadenylation. Appropriate transcriptional terminators and those which are known to function in plants include the CaMV 35S terminator, the tml terminator, the nopaline synthase terminator, the pea rbcS E9 terminator. These can be used in both monocotyledons and dicotyledons.

Sequences for the Enhancement or Regulation of Expression

Numerous sequences have been found to enhance gene expression from within the transcriptional unit and these sequences can be used in conjunction with the genes of this invention to increase their expression in transgenic plants.

Various intron sequences have been shown to enhance expression, particularly in monocotyledonous cells. For example, the introns of the maize Adh1 gene have been found to significantly enhance the expression of the wild-type gene under its cognate promoter when introduced into maize cells. Intron 1 was found to be particularly effective and enhanced expression in fusion constructs with the chloramphenicol acetyltransferase gene (Callis et al., *Genes Develop.* 1: 1183-1200 (1987)). In the same experimental system, the intron from the maize bronze1 gene had a similar effect in enhancing expression (Callis et al., *supra*). Intron sequences have been routinely incorporated into plant transformation vectors, typically within the non-translated leader.

A number of non-translated leader sequences derived from viruses are also known to enhance expression. Specifically, leader sequences from Tobacco Mosaic Virus (TMV, the "W-sequence"), Maize Chlorotic Mottle Virus (MCMV), and Alfalfa Mosaic Virus (AIMV) have been shown to be effective in enhancing expression (e.g. Gallie et al. *Nucl. Acids Res.* 15: 8693-8711 (1987); Skuzeski et al. *Plant Molec. Biol.* 15: 65-79 (1990)).

Example 3: Transformation of Monocotyledons

Transformation of monocotyledon species such as wheat or maize has become routine. Preferred techniques include direct gene transfer into protoplasts using PEG or electroporation techniques, and particle bombardment into callus tissue. Transformations can be undertaken with a single DNA species or multiple DNA species (i.e. co-transformation) and both these techniques are suitable for use with this invention. Co-transformation may have the advantage of avoiding complex vector construction and of generating transgenic plants with unlinked loci for the gene of interest and the selectable marker, enabling the removal of the selectable marker in subsequent generations, should this be regarded desirable. However, a disadvantage of the use of co-transformation is the less than 100% frequency with which separate DNA species are integrated into the genome (Schocher et al. *Biotechnology* 4: 1093-1096 (1986)).

EP-A-292 435 (to Ciba-Geigy), EP-A-392 225 (to Ciba-Geigy) and WO 93/07278 (to Ciba-Geigy) describe techniques for the preparation of callus and protoplasts from an elite

inbred line of maize, transformation of protoplasts using PEG or electroporation, and the regeneration of maize plants from transformed protoplasts. Gordon-Kamm et al., *Plant Cell* 2: 603-618 (1990)) and Fromm et al., *Biotechnology* 8: 833-839 (1990)) have published techniques for transformation of A188-derived maize line using particle bombardment. Furthermore, WO 93/07278 (to Ciba-Geigy) and Koziel et al., *Biotechnology* 11: 194-200 (1993) describe techniques for the transformation of elite inbred lines of maize by particle bombardment. This technique utilizes immature maize embryos of 1.5-2.5 mm length excised from a maize ear 14-15 days after pollination and a PDS-1000He Biolistics device for bombardment.

Transformation of rice can also be undertaken by direct gene transfer techniques utilizing protoplasts or particle bombardment. Protoplast-mediated transformation has been described for Japonica-types and Indica-types (Zhang et al., *Plant Cell Rep* 7: 379-384 (1988); Shimamoto et al. *Nature* 338: 274-277 (1989); Datta et al. *Biotechnology* 8: 736-740 (1990)). Both types are also routinely transformable using particle bombardment (Christou et al. *Biotechnology* 9: 957-962 (1991)).

EP-A-332 581 (to Ciba-Geigy) describes techniques for the generation, transformation and regeneration of Pooideae protoplasts. These techniques allow the transformation of *Dactylis* and wheat. Furthermore, wheat transformation has been described by Vasil et al., *Biotechnology* 10: 667-674 (1992)) using particle bombardment into cells of type C long-term regenerable callus, and also by Vasil et al., *Biotechnology* 11: 1553-1558 (1993)) and Weeks et al., *Plant Physiol.* 102: 1077-1084 (1993) using particle bombardment of immature embryos and immature embryo-derived callus. A preferred technique for wheat transformation, however, involves the transformation of wheat by particle bombardment of immature embryos and includes either a high sucrose or a high maltose step prior to gene delivery. Prior to bombardment, any number of embryos (0.75-1 mm in length) are plated onto MS medium with 3% sucrose (see Murashige & Skoog, *Physiologia Plantarum* 15: 473-497 (1962)) and 3 mg/l 2,4-D for induction of somatic embryos which is allowed to proceed in the dark. On the chosen day of bombardment, embryos are removed from the induction medium and placed onto the osmoticum (i.e. induction medium with sucrose or maltose added at the desired concentration, typically 15%). The embryos are allowed to plasmolyze for 2-3 h and are then bombarded. Twenty embryos per target plate is typical, although not critical. An appropriate gene-carrying plasmid (such as pCIB3064 or pSG35) is precipitated onto micrometer size gold particles using standard procedures. Each plate of embryos is shot with the DuPont Biolistics helium device using a burst pressure of ~1000 psi using a

standard 80 mesh screen. After bombardment, the embryos are placed back into the dark to recover for about 24 h (still on osmoticum). After 24 hours, the embryos are removed from the osmoticum and placed back onto induction medium where they stay for about a month before regeneration. Approximately one month later the embryo explants with developing embryonic callus are transferred to regeneration medium (MS + 1 mg/liter NAA, 5 mg/liter GA), further containing the appropriate selection agent (10 mg/l basta in the case of pCIB3064 and 2 mg/l methotrexate in the case of pSOG35). After approximately one month, developed shoots are transferred to larger sterile containers known as "GA7s" which contained half-strength MS, 2% sucrose, and the same concentration of selection agent.

Example 4: MDMV-B Resistance Conferred by Expression of Translationally Altered Viral Transcripts

Our research has focused on cloning and sequencing the remainder of the MDMV-B genome. We have disclosed the majority of the MDMV-B sequence in this application. We have identified coding regions within the MDMV-B coding region based on conserved motifs previously identified in other potyviruses. The regions of the virus selected for use as transgenes have been the MDMV-B non-structural proteins (i.e. Replicase, Proteinase, and Helicase). These regions were targeted based on the expected higher degree of sequence conservation within these genes among strains of MDMV. We predict that the use of these regions will give the highest probability of obtaining resistance to multiple strains of MDMV when transformed into elite maize inbreds. The sequences have been used to transform maize plants for the purpose of conferring virus resistance.

Maize dwarf mosaic virus strain B (MDMV-B) was obtained from Dr. S. Jensen (University of Nebraska-Lincoln) and maintained in a susceptible maize inbred by serial inoculation. Virus was prepared for inoculation as previously described (see Law, M. D. et al. *Phytopathology* 79:757-761, (1988)).

The virus was purified from two week old infected maize tissue by the following protocol. The harvested tissue was homogenized with 0.2 sodium acetate, pH 5.0 containing 0.1% b-mercaptoethanol (1:6 ratio W:V) in a blender. The homogenate was filtered through cheesecloth and then centrifuged for 15 minutes at 6000 RPM (Sorvall GSA rotor). The recovered supernatant was then filtered through glass wool and adjusted to a concentration of 0.5% Triton X-100 and 0.2M NaCl. The virus was precipitated from the

solution by adding PEG 8000 (8% final concentration) and then stirring for 2 hours at 4°C. The virus was recovered by centrifugation for 15 minutes at 8,000 RPM (Sorvall GSA rotor).

The resulting pellet containing the virus was resuspended by stirring in 0.1M Tris pH 6.5 containing 0.032 M sodium citrate. The virus solution was clarified by centrifugation through a 20% sucrose pad for 2 hours at 28,000 RPM (SW28 rotor). The recovered pellet was resuspended in 10 ml of 0.1M Tris pH 6.5 containing 0.032 M sodium citrate. The supernatant was adjusted to a concentration of 34% cesium sulfate and centrifuged for 14 hours at 48,000 RPM (Ti 70.1 rotor). The opalescent band containing the virus was removed and dialyzed against 0.1M Tris pH 6.5 containing 0.032 M sodium citrate. Viral RNA was isolated from the purified virions by phenol extraction and ethanol precipitation.

The isolated RNA was then used as template for cDNA preparation using oligo dT primers. The preparation of cDNA clones were performed by standard procedures as described (see Sambrook, J. et al., *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, (1989)).

Constructs were prepared to specific regions of the MDMV-B genome by PCR amplification from cDNA clones. The region amplified by PCR was typically 1200 to 1400 nucleotides in length and was confirmed by sequencing. Constructs were prepared to the regions of the MDMV-B genome which encode the viral replicase (NIb), proteinase (NIa), and cylindrical inclusion protein (CIP). These regions were selected based on the higher sequence conservation within these regions between members of the potyvirus family. The constructs corresponding to a specific viral coding region were altered during PCR amplification by nucleotide substitutions within the primers. A methionine translation initiation codon was generated at the first codon preceding the first native codon and a termination codon was created at the seventh codon in all constructs tested. This would create a mRNA only capable of translating small peptides. The constructs were then ligated into either the pUBA plasmid (see Toki et al. *Plant Physiol.* 100:1503-1507, (1992)) or the pCIB4421 plasmid. The pUBA plasmid contained the Ubiquitin promoter and the NOS terminator while pCIB4421 contained the maize phosphoenolpyruvate carboxylase (PEPC) promoter and the 35S terminator. The plasmid constructs were then verified by DNA sequencing.

The constructs used in this example to transform maize plants have been designated pCIB5018 and pCIB 5019. pCIB5018 was constructed by ligating the PCR amplified NIa fragment (nucleotides 4452 -5744 of SEQ ID No. 1) into pCIB4421. The NIa fragment used for ligation had previously been altered by insertion of an ATG codon immediately before

the first nucleotide of the first codon (i.e. the G at position 4452 of SEQ ID No. 1) and substitution of a thymidine (T) for the adenine (A) at nucleotide 4470 of SEQ ID No. 1 to create a premature stop codon. pCIB5019 was constructed by ligating the altered Nla fragment described above into the pUBA plasmid.

Microprojectile Bombardment Protocols

Plasmid DNA was precipitated onto 1mm gold microcarrier particles as described in the DuPont Biolistic manual. 5mg of plasmid DNA containing a synthetic phosphinothrycin acetyltransferase selectable marker gene and 5mg of either pCIB5018 or pCIB5019 were added per 50ml of prepared microcarrier. The synthetic phosphinothrycin acetyltransferase selectable marker gene provides resistance to the same selection agents as the BAR gene (see Kramer, C. et al. *Planta* 190: 454-458 (1993)). Bombardment of tissue was carried out with the DuPont PDS-1000He Biolistic® device. An additional 150x150mesh/linear inch screen was inserted 2cm below the stopping screen. Immature embryos were bombarded with 1550psi rupture discs on a plate angled 6-8cm below the stopping screen to maximize scutellum exposure to particles. Type I callus was placed 4cm below the stopping screen and 900psi rupture discs were used in bombardment. All plates for both explant types were bombarded twice.

Immature Embryo Explant Source Initiation and Selection

Immature embryos of a proprietary Ciba elite line (CG00526) were used as the initial explant source in microprojectile-mediated transformation. Embryos were excised from the ears 10-14 days post-pollination, when 1-2mm in length. After surface sterilization in a 10% Clorox solution, embryos were plated embryonic axis down on the surface of the agar-solidified medium. Embryos were plated onto Duncan's "D" callus induction medium plus 5mg/l chloramben, 2% sucrose, 12mM proline and either the organic amendments specified in Duncan's (2DG4) or a modified version (2DA1) which omits the casein hydrolysate and adds the amino acids minus glutamine and asparagine from Kao and Michayluk's "KM" medium (see Kao and Michayluk, *Planta* 126:105-110, (1975)). The plated embryos were kept in a 25_C dark culture room continuously until the regeneration phase was initiated. The day after plating the embryos were transferred to the appropriate G4 or A1 media containing 12% sucrose at least four hours prior to microprojectile bombardment. Thirty-six embryos were arranged in a 2-3 cm circle in the center of the plate. The embryos remain on the 12% sucrose plate overnight after bombardment. The following day, embryos were

transferred either to 2DG4 + 5 chloramben + the equivalent of a 10mg/l concentration of Basta[®] herbicide (glufosinate ammonium) or 2DA1 + 5 chloramben + 5mg/l Basta.

Fourteen days from the initial excision and plating, developing compact, organized type I callus was excised from the original explant and subcultured to either 2DG4 + 0.5mg/l 2,4-D + 10mg/l Basta or 2DA1 + 0.5mg/l 2,4-D + 5mg/l Basta. Viable, healthy callus was serially subcultured every fourteen days during the selection phase. All tissue was then transferred to Duncan's medium, modified by omitting all amino acids, plus 2% sucrose, 0.5mg/l 2,4-D and 10mg/l Basta (2DG8) at the end of eight weeks. After a two week passage on the G8 medium, all living tissue was transferred to regeneration medium.

Type I Explant Source Initiation and Selection

Immature embryos of the Ciba elite line (CG00526) were plated embryonic axis down onto 2DG4 + 5 chloramben at the 1-2mm length size. The developing compact, highly organogenic (type I) callus was excised from the original embryo explant after fourteen days and maintained serially on 2DG4 + 0.5mg/l 2,4-D by subculturing to fresh medium every ten-fourteen days. When the callus lines obtained were two to three months old, they were prepared for microprojectile bombardment. The tissue was subcultured to fresh medium in small pieces approximately 1-3mm in size one to two days prior to bombardment. On the day of bombardment, the tissue was arranged in a 2-3cm circle in the center of a DA1 plate containing 12% sucrose and 0.5mg/l 2,4-D at least four hours prior to bombardment. The callus was kept on the plate after bombardment overnight, and transferred the next day to 2DA1 + 0.5mg/l 2,4-D + 10mg/l Basta. Viable, healthy callus was serially subcultured on the same medium every fourteen days during the selection phase. All tissue was transferred to Duncan's medium, modified by omitting all amino acids, plus 2% sucrose, 0.5mg/l 2,4-D and 10mg/l Basta (2DG8) at the end of eight weeks. After a two week passage on the G8 medium, all living tissue was transferred to regeneration medium.

Regeneration and Plantlet Establishment of Immature Embryo and Type I Explant Source Experiments

Tissue for regeneration was moved to a 25°C light culture room under a 16 hour photoperiod. Regeneration medium consisted of Murashige and Skoog's (MS) salts and vitamins, 3% sucrose + 0.25mg/l ancymidol, 1.0mg/l NAA, 0.5mg/l kinetin and 5mg/l Basta.

After a two week passage on the regeneration medium with growth regulators, the tissue was transferred to MS medium + 3mg/lBasta and no additional growth regulators. Plantlets reaching 1-3cm length were transferred from plates to Magenta[®]GA-7 boxes containing MS medium (0.75X concentration+ 1% sucrose) and no Basta for root development. Plantlets with sufficient root development were transplanted to soil and moved to the greenhouse. Plantlets were hardened off in a 70% humidity phytotron for one to two weeks before moving the plants to the greenhouse range. The greenhouse conditions were as follows: 55% humidity, 400 Einsteins light intensity, 16 hour photoperiod, 80-84_F Day temperature, 64-68_F Night temperature. Plants were allowed to grow to maturity in the greenhouse and were either selfed or backcrossed to the parental line in the T₁ generation.

Analysis of T₀ Plants

T₀ plantlets were first assayed by polymerase chain reaction (PCR) to detect the selectable marker, the gene of interest and an alcohol dehydrogenase (Adh) gene sequence as an internal assay control. Plantlets were assayed at approximately eight to fourteen cm height, when the plantlets were still in the GA-7 boxes. Standard PCR conditions were used (see Kramer, C. et al. *Planta* 190: 454-458 (1993)). The Adh internal control primer pair sequence was TGCATGTCGGTTGTGTTGCA (SEQ ID NO. 3) and CTCAGCAAGTACCTAGACCA (SEQ ID No. 4). The primer pair sequence for the synthetic PAT gene was TGTCTCCGGAGAGGAGACC' (SEQ ID No. 5) and CCAACATCATGCCATCCACC (SEQ ID No. 6). The primer pair sequence for the Nla proteinase gene is GCGGGATCCATGGGGAAGAACAACGCAGTTGA (5') (SEQ ID No. 7) and GCGGAGCTCTTACTCTTCAACGCTCGCGTC (3') (SEQ ID No. 8). The parameters for PCR amplification for all primer pairs were 45 sec at 94 _C, 30 sec at 62_C, 30 sec at 72_C plus a 3 sec/cycle extension elongation for 40 cycles.

Plantlets identified by PCR to be transformed were analyzed by Northern blot assay for mRNA transcript of the gene of interest (Nla proteinase). Plants were assayed for mRNA expression either while in the GA-7 containers or when the plants had been acclimated in the greenhouse. The probe was a 1303 bp fragment of the Nla gene excised by a BamH1/SacI restriction digest of the pCIB5019 plasmid. Labeling was carried out with the Gibco/BRL RadPrime DNA Labeling kit as described by the manufacturer. Northern blot protocols were performed as described (see Sambrook, J. et al., "Molecular Cloning: A Laboratory Manual", Cold Spring Harbor Laboratory Press, (1989)).

Analysis of T₁ Plants

T₁ seed harvested from the T₀ plants was first dried down in the drying room for one to two weeks before planting. Seed were planted directly in flats and watered in. The flats were bottom watered with either a 0.15% volume/volume Basta solution or with water two days after planting. Four different transformation events were tested for herbicide and disease resistance in this example, as well as the wild-type elite control. Forty seeds from each individual transformed plant were tested initially, 20 in Basta and 20 in the water control. Seven days after the first Basta drench, a second drench was carried out in the same manner.

All plants were inoculated with MDMV-B following the second Basta soil drench when the plants were 4-5 inches in height (3-5 Leaf Stage). A second virus inoculation was performed on all plantlets 4-6 days after the first inoculation to insure infection. Plants were scored for viability in the plus and minus Basta drench and for the presence or absence of viral symptoms at the end of two and a half weeks.

Plants which showed resistance to the virus, as measured by the absence of viral symptoms, and a susceptible sibling were assayed by Northern blot analysis using the Nla fragment as described above. The resistant plants were also assayed by ELISA and Western blot analysis for the presence of MDMV-B coat protein in the plants.

ELISA and Western Blot analysis of the transgenic plants.

The primary antibody used for both assays was a polyclonal antibody specific for the MDMV-B coat protein which was obtained from Dr. S. Jensen (University of Nebraska-Lincoln). The second antibody was an affinity purified polyclonal IgG alkaline phosphatase labeled goat anti-rabbit antibody (Kirkegard and Perry Laboratories, Gaithersburg, Maryland).

ELISA Analysis

Tissue samples were taken from all plants not exhibiting characteristic MDMV-B symptoms and from one infected plant. Samples were also taken from healthy and infected CG00526 plants as controls. The samples (two leaf punches-1 cm in diameter) were taken from both the inoculated leaf and the youngest available leaf. The tissue samples were homogenized in 0.400 ml of borate buffered saline (100mM boric acid, 25mM sodium

borate, 75mM sodium chloride). Aliquots (50ml) of each sample were applied to a ethanol washed ELISA plate and incubated overnight at 4°C. The plates were then washed once with ELISA wash buffer (10mM Tris-HCl, 0.05% Tween-20, 0.02% sodium azide), and blocked with ELISA block/diluent (10mM sodium phosphate, 140mM sodium chloride, 0.05% Tween-20, 1% BSA, 0.02% sodium azide) for one hour at room temperature. The plates were washed three times with ELISA wash buffer. The primary antibody was applied at a 1:5000 dilution in 50ml of ELISA block/diluent and incubated for 2 hours at 37°C and then washed three times with ELISA wash buffer. The second antibody was applied at a concentration of 1.5mg/ml in ELISA block/diluent and incubated for 2 hours at 37°C. The plates were washed three times with ELISA wash buffer and were developed by incubation in ELISA substrate (Kirkegard and Perry) for 30 minutes at room temperature. The reaction was stopped by the addition of 50ml of 3M sodium hydroxide. The plates were read with a SLT 340 ATTC ELISA plate reader (SLT Labinstruments) at 405nm.

Western Blot Analysis

Western blot analysis was performed on samples used for ELISA analysis. A 2ml aliquot of the samples was diluted into 10ml of 1X loading dye (Novex Inc). The samples were electrophoresed on an 8%-16% Tris-glycine polyacrylamide gel (Novex) in Tris running buffer (25mM Tris-Base, 192mM glycine, 0.1%SDS) at 120 volts for approximately 2.5 hours. The gel was blotted onto nitrocellulose using a Biorad blotting apparatus in transfer buffer (25mM Tris-Base, 192mM glycine, and 10% methanol) at 120 volts for 45 minutes. The filter was blocked with blocking/diluent (1X TBS, (20mM Tris-Base, 500mM NaCl, pH 7.5), 0.05% Tween-20, 1% BSA, 5% lamb serum) at room temperature for 45 minutes. The filter was incubated with the primary antibody, described above, at a dilution of 1:1000 in blocking/diluent at room temperature for 1.25 hours. The filter was washed for five minutes in 1XTTBS, (1X TBS, 0.05% Tween-20). The second antibody, described above, was incubated with the filter in blocking/diluent at a dilution of 1:1000, for 1.25 hours at room temperature. The filters were washed twice for 5 minutes in 1XTTBS followed by a single wash in 1XTBS for 5 minutes. The filter was developed with Nitro Blue Tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphatase (BCIP) in 0.1M Tris-HCl pH 9.5 as described by the manufacturer. The filter was developed for approximately 20 minutes and then stopped by washing the filter with water.

Characterization of the MDMV-B Genome

Clones have been isolated and sequenced representing 8530 nucleotides of the MDMV-B genome. We have identified a single large open reading frame as would be expected of a virus belonging to the potyvirus family. We have identified regions of the polyprotein which would encode the coat protein (nucleotides 7308-8291 of SEQ ID No. 1 and amino acids 2436-2763 of SEQ ID No. 2), the putative RNA dependent RNA polymerase (RdRp) termed NIb (nucleotides 5745-7307 of SEQ ID No. 1 and amino acids 1915-2435 of SEQ ID No. 2), the NIa proteinase (nucleotides 4452-5744 of SEQ ID No. 1 and amino acids 1484-1914 of SEQ ID No. 2), the 6K protein (nucleotides 4293-4451 of SEQ ID No. 1 and amino acids 1431-1483 of SEQ ID No. 2), cylindrical inclusion protein (CIP) containing the helicase (nucleotides 2376-4292 of SEQ ID No. 1 and amino acids 792-1430 of SEQ ID No. 2), P3 proteinase (nucleotides 1134-2375 of SEQ ID No. 1 and amino acids 378-791 of SEQ ID No. 2), and a portion of the helper component-P2 proteinase (HC-Pro) (nucleotides 3-1133 of SEQ ID No. 1 and amino acids 1-377 of SEQ ID No. 2). Identification was based on the location of putative cleavage sites and conserved motifs. The MDMV-B sequence of the CP region from our isolate was 99% identical to the previously sequenced MDMV-B CP and 78% identical to the MDMV-A CP. Further comparisons could not be made due to the lack of additional sequence to other MDMV strains. The sequence of MDMV-B was then compared to other potyviruses and was found to exhibit approximately 60% nucleotide sequence identity to other potyviruses. The level of identity varied little when sequences encoding the different proteins were used for the comparison.

T₀ Analysis

Eighteen lines (individual transformation events from selection and regeneration) were obtained from the experiments in this example. 17 of the 18 lines were positive by PCR for the selectable marker, and 14 for the gene of interest. All 14 events which were PCR positive for the NIa gene were also positive for expression in the Northern analysis. The predominate mRNA species was approximately 1300 nucleotides in length which would correspond to the predicted size of the transgene. A smaller species approximately 1000 nucleotides in length was also detected which most likely arose by processing. Differences in mRNA expression levels were seen between different events as well as between

individual plants (siblings) from a given event. All PCR positive plants were used for seed production (T₁).

T₁ Analysis

Four plants from two different events were identified to be resistant to the virus inoculation as evidenced by the absence of visual symptoms. There was no correlation to Basta tolerance in this example. Northern analysis of the four plants showed no detectable Nla transcript in the four resistant plants, while an infected sibling plant from the same original ear (T₀) was shown to have high levels of viral RNA. The levels of MDMV-B in the infected sibling was similar to the levels seen in the control CG00526 plants.

The resistant plants were also evaluated for the presence of viral coat protein by ELISA.

The four values obtained for each sample, duplicate samples from the inoculated leaf and non-inoculated leaf, were averaged and a comparison made against the infected and healthy controls. No detectable virus was present in the resistant transformed plant lines by ELISA at which the threshold of detection was approximately 2 ng of virus per sample. In contrast, the transformed siblings which exhibited symptoms contained levels of virus similar to that seen in the infected CG00526 control plants. These results show conclusive evidence that the four plants were immune to MDMV-B infection (i.e. not supporting virus replication). The resistance was durable in that the resistant plants withstood two inoculations with high MDMV-B inoculum concentrations. The inoculum concentrations used in these experiments typically result in symptoms within four days in susceptible plant lines. Yet, the resistant plants have not produced visible symptoms nor detectable virus six weeks following inoculation.

Various modifications of the invention described herein will become apparent to those skilled in the art. Such modifications are intended to fall within the scope of the appended claims.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:

- (A) NAME: CIBA-GEIGY AG
- (B) STREET: Klybeckstr. 141
- (C) CITY: Basel
- (E) COUNTRY: Switzerland
- (F) POSTAL CODE (ZIP): 4002
- (G) TELEPHONE: +41 61 69 11 11
- (H) TELEFAX: + 41 61 696 79 76
- (I) TELEX: 962 991

(ii) TITLE OF INVENTION: USE OF TRANSLATIONALLY ALTERED RNA TO CONFER
RESISTANCE TO MAIZE DWARF MOSAIC VIRUS AND OTHER
MONOCOTYLEDONOUS PLANT VIRUSES

(iii) NUMBER OF SEQUENCES: 8

(iv) COMPUTER READABLE FORM:

- (A) MEDIUM TYPE: Floppy disk
- (B) COMPUTER: IBM PC compatible
- (C) OPERATING SYSTEM: PC-DOS/MS-DOS
- (D) SOFTWARE: PatentIn Release #1.0, Version #1.30B

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 8543 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: RNA (genomic)

(iii) HYPOTHETICAL: NO

(ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION: 3..8291

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(D) OTHER INFORMATION: /product= "polyprotein encoded by
MMV-B genome"

(ix) FEATURE:

(A) NAME/KEY: 3'UTR
(B) LOCATION: 8292..8530

(ix) FEATURE:

(A) NAME/KEY: misc_RNA
(B) LOCATION: 3..1133
(D) OTHER INFORMATION: /product= "3-prime sequence for
HC-Pro"

(ix) FEATURE:

(A) NAME/KEY: misc_RNA
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(D) OTHER INFORMATION: /product= "P3 proteinase"

(ix) FEATURE:

(A) NAME/KEY: misc_RNA
(B) LOCATION: 2376..4292
(D) OTHER INFORMATION: /product= "cylindrical inclusion
protein"

(ix) FEATURE:

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(D) OTHER INFORMATION: /product= "K2 (6kD protein)"

(ix) FEATURE:

(A) NAME/KEY: misc_RNA
(B) LOCATION: 4452..5744
(D) OTHER INFORMATION: /product= "N1a proteinase"

(ix) FEATURE:

(A) NAME/KEY: misc_RNA
(B) LOCATION: 5745..7307
(D) OTHER INFORMATION: /product= "N1b replicase"

(ix) FEATURE:

(A) NAME/KEY: misc_RNA
(B) LOCATION: 7308..8291
(D) OTHER INFORMATION: /product= "coat protein"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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1					5					10					15	
AGA	AUG	AUA	CAA	UUU	AUC	AAA	GAA	AGG	UGC	AAU	CCA	AAA	UUU	UCG	CAU	95
Arg	Met	Ile	Gln	Phe	Ile	Lys	Glu	Arg	Cys	Asn	Pro	Lys	Phe	Ser	His	
					20					25					30	

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ACG GUU UAU UGG UUU AUA CCU GAU AUA UUU AGA CUC GUG CAC AUA UUC			1919
Thr Val Tyr Trp Phe Ile Pro Asp Ile Phe Arg Leu Val His Ile Phe			
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AAA GUG GAU GGC CGA ACA AUG AAA CAA AAC ACA ACA GGA AUC AUU ACA Lys Val Asp Gly Arg Thr Met Lys Gln Asn Thr Thr Gly Ile Ile Thr 1075 1080 1085	3263
AAC GGU ACC GCA CAA AAG AAG UGU UUU GUU GUC GCA ACG AAU AUA AUU Asn Gly Thr Ala Gln Lys Lys Cys Phe Val Val Ala Thr Asn Ile Ile 1090 1095 1100	3311
GAG AAU GGC GUC ACA CUA GAU AUU GAU GUU GGU GUC GAC UUC GGA CUU Glu Asn Gly Val Thr Leu Asp Ile Asp Val Gly Val Asp Phe Gly Leu 1105 1110 1115	3359
AAA GUC UCA GCU GAC UUG GAC GUU GAC AAC AGG GCG GUA UUG UAU AAA Lys Val Ser Ala Asp Leu Asp Val Asp Asn Arg Ala Val Leu Tyr Lys 1120 1125 1130 1135	3407

CGC GUA AGU AUA UCA UAU GGU GAA CUC AUA CAA CGA UUG GGU CGU GUU Arg Val Ser Ile Ser Tyr Gly Glu Leu Ile Gln Arg Leu Gly Arg Val 1140 1145 1150	3455
GGC AGA AAU AAA CCU GGU ACA GUU AUU CGA AUC GGA AAA ACA AUG AAA Gly Arg Asn Lys Pro Gly Thr Val Ile Arg Ile Gly Lys Thr Met Lys 1155 1160 1165	3503
GGU UUG CAG GAA AUU CCA GCA AUG AUC GCA ACA GAG GCA GCC UUC AUG Gly Leu Gln Glu Ile Pro Ala Met Ile Ala Thr Glu Ala Ala Phe Met 1170 1175 1180	3551
UGU UUC GCU UAC GGU CUU AAA GUU AUC ACU CAU AAU GUU UCA ACG ACC Cys Phe Ala Tyr Gly Leu Lys Val Ile Thr His Asn Val Ser Thr Thr 1185 1190 1195	3599
CAU CUU GCA AAG UGC ACA GUU AAA CAA GCG AGA ACC AUG AUG CAA UUU His Leu Ala Lys Cys Thr Val Lys Gln Ala Arg Thr Met Met Gln Phe 1200 1205 1210 1215	3647
GAA UUA UCA CCA UUU GUC AUG GCU GAG CUC GUU AAG UUU GAU GGU UCA Glu Leu Ser Pro Phe Val Met Ala Glu Leu Val Lys Phe Asp Gly Ser 1220 1225 1230	3695
AUG CAU CCA CAA AUA CAU GAG GCA CUA GUA AAA UAC AAA CUU AGA GAU Met His Pro Gln Ile His Glu Ala Leu Val Lys Tyr Lys Leu Arg Asp 1235 1240 1245	3743
UCU GUC AUA AUG CUC AGA CCG AAU GCA CUU CCA AGG GUC AAU UUA CAU Ser Val Ile Met Leu Arg Pro Asn Ala Leu Pro Arg Val Asn Leu His 1250 1255 1260	3791
AAU UGG CUU ACA GCC CGA GAU UAU AAU AGA AUA GGA UGU UCA UUA GAA Asn Trp Leu Thr Ala Arg Asp Tyr Asn Arg Ile Gly Cys Ser Leu Glu 1265 1270 1275	3839
CUC GAA GAC CAC GUC AAA AUU CCG UAC UAC AUU AGG GGA GUU CCU GAC Leu Glu Asp His Val Lys Ile Pro Tyr Tyr Ile Arg Gly Val Pro Asp 1280 1285 1290 1295	3887
AAG UUG UAU GGA AAG CUA UAU GAU AUU AUC UUA CAG GAU AGU CCA ACU Lys Leu Tyr Gly Lys Leu Tyr Asp Ile Ile Leu Gln Asp Ser Pro Thr 1300 1305 1310	3935
AGU UGC UAC AGU AGA CUA UCA AGU GCG UGU GCA GGU AAA GUA GCA UAU Ser Cys Tyr Ser Arg Leu Ser Ser Ala Cys Ala Gly Lys Val Ala Tyr 1315 1320 1325	3983
ACU CUG CGA ACU GAU CCA UUU UCA CUU CCA AGA ACA AUA GCA AUA AUU Thr Leu Arg Thr Asp Pro Phe Ser Leu Pro Arg Thr Ile Ala Ile Ile 1330 1335 1340	4031
AAU GCC UYA AUC ACG GAG GAG UAU GCG AAG AGA GAU CAC UAU CGU AAC Asn Ala Xaa Ile Thr Glu Glu Tyr Ala Lys Arg Asp His Tyr Arg Asn	4079

1345	1350	1355	
AUG AUU YCA AAC CCA UCU UCA UCA CAC GCA UUC UCA CUC AAU GGG UUG			4127
Met Ile Xaa Asn Pro Ser Ser Ser His Ala Phe Ser Leu Asn Gly Leu			
1360	1365	1370	1375
GUG UCU AUG AUC GCU ACU AGA UAU AUG AAA GAC CAC ACA AAG GAG AAU			4175
Val Ser Met Ile Ala Thr Arg Tyr Met Lys Asp His Thr Lys Glu Asn			
1380	1385		1390
AUU GAC AAA CUC AUC AGA GUG CGU GAU CAA UUA CUU GAG UUU CAA GGU			4223
Ile Asp Lys Leu Ile Arg Val Arg Asp Gln Leu Leu Glu Phe Gln Gly			
1395	1400		1405
ACU GGA AUG CAA UUU CAA GAU CCA UCA GAA CUC AUG GAA AUU GGG GCU			4271
Thr Gly Met Gln Phe Gln Asp Pro Ser Glu Leu Met Glu Ile Gly Ala			
1410	1415		1420
CUC AAC ACA GUU AUU CAC CAA GGA AUG GAC GCA AUU GCA GCU UGU AUU			4319
Leu Asn Thr Val Ile His Gln Gly Met Asp Ala Ile Ala Ala Cys Ile			
1425	1430		1435
GAG UUA CAA GGA CGA UGG AAU GCU UCA CUU AUA CAA CGC GAU CUC CUA			4367
Glu Leu Gln Gly Arg Trp Asn Ala Ser Leu Ile Gln Arg Asp Leu Leu			
1440	1445		1450
AUU GCA GGU GGA GUU UUU AUC GGA GGC AUU UUG AUG AUG UGG AGC CUA			4415
Ile Ala Gly Gly Val Phe Ile Gly Gly Ile Leu Met Met Trp Ser Leu			
1460	1465		1470
UUU ACU AAA UGG AGU AAC ACA AAU GUC UCA CAU CAG GGG AAG AAC AAA			4463
Phe Thr Lys Trp Ser Asn Thr Asn Val Ser His Gln Gly Lys Asn Lys			
1475	1480		1485
CGC AGU AGA CAA AAA CUU CGA UUC AAA GAA GCA AGA GAC AAC AAA UAU			4511
Arg Ser Arg Gln Lys Leu Arg Phe Lys Glu Ala Arg Asp Asn Lys Tyr			
1490	1495		1500
GCA UAU GAU GUC ACA GGA UCG GAA GAA UGC CUU GGC GAG AAU UUU GGA			4559
Ala Tyr Asp Val Thr Gly Ser Glu Glu Cys Leu Gly Glu Asn Phe Gly			
1505	1510		1515
ACA GCC UAU ACA AAG AAA GGU AAA GGA AAA GGA ACU AAA GUU GGA CUC			4607
Thr Ala Tyr Thr Lys Lys Gly Lys Gly Lys Gly Thr Lys Val Gly Leu			
1520	1525		1530
GGU GUG AAG CAG CAU AAA UUC CAU AUG AUG UAC GGU UUC GAU CCC CAA			4655
Gly Val Lys Gln His Lys Phe His Met Met Tyr Gly Phe Asp Pro Gln			
1540	1545		1550
GAG UAC AAC CUA AUU CGG UUU GUC GAU CCA CUC ACG GGA GCA ACU CUU			4703
Glu Tyr Asn Leu Ile Arg Phe Val Asp Pro Leu Thr Gly Ala Thr Leu			
1555	1560		1565
GAU GAA CAA AUC CAU GCC GAU AUA CGC UUA AUU CAA GAG CAC UUC GCU			4751

Asp Glu Gln Ile His Ala Asp Ile Arg Leu Ile Gln Glu His Phe Ala 1570 1575 1580	
GAA AUU CGU GAG GAG GCA GUG AUU AAU GAC ACA AUU GAA AGG CAG CAG Glu Ile Arg Glu Glu Ala Val Ile Asn Asp Thr Ile Glu Arg Gln Gln 1585 1590 1595	4799
AUU UAC GGC AAU CCU GGA CUA CAA GCA UUU UUC AUA CAA AAU GGG UCA Ile Tyr Gly Asn Pro Gly Leu Gln Ala Phe Phe Ile Gln Asn Gly Ser 1600 1605 1610 1615	4847
GCA AAC GCU CUG AGA GUU GAU UUA ACA CCA CAU UCA CCU ACA CGA GUU Ala Asn Ala Leu Arg Val Asp Leu Thr Pro His Ser Pro Thr Arg Val 1620 1625 1630	4895
GUC ACA GGU AAU AAC AUA GCA GGG UUC CCA GAA UAU GAA GGA ACA CUU Val Thr Gly Asn Asn Ile Ala Gly Phe Pro Glu Tyr Glu Gly Thr Leu 1635 1640 1645	4943
CGU CAG ACU GGA ACA GCU AUA ACU AUA CCC AUU GGU CAA GUC CCA AUC Arg Gln Thr Gly Thr Ala Ile Thr Ile Pro Ile Gly Gln Val Pro Ile 1650 1655 1660	4991
GCA AAU GAA GCA GGG GUU GCA CAC GAG UCA AAA UCC AUG AUG AAC GGG Ala Asn Glu Ala Gly Val Ala His Glu Ser Lys Ser Met Met Asn Gly 1665 1670 1675	5039
UUG GGU GAU UAC ACA CCA AUA UCG CAA CAA UUG UGU CUA GUA CAA AAU Leu Gly Asp Tyr Thr Pro Ile Ser Gln Gln Leu Cys Leu Val Gln Asn 1680 1685 1690 1695	5087
GAC UCG GAU GGG GUA AAG CGG AAU GUA UUU UCU AUU GGA UAU GGC UCA Asp Ser Asp Gly Val Lys Arg Asn Val Phe Ser Ile Gly Tyr Gly Ser 1700 1705 1710	5135
UAU CUU AUU UCA CCA GCG CAC UUA UUC AAA UAC AAC AAU GGU GAA AUA Tyr Leu Ile Ser Pro Ala His Leu Phe Lys Tyr Asn Asn Gly Glu Ile 1715 1720 1725	5183
ACA AUU AGA UCA UCA AGA GGA UUG UAC AAA AUU CGU AAU UCU GUG GAU Thr Ile Arg Ser Ser Arg Gly Leu Tyr Lys Ile Arg Asn Ser Val Asp 1730 1735 1740	5231
UUA AAA UUA CAU CCA AUU GCA CAC AGA GAC AUG GUC AUA AUU CAA CUC Leu Lys Leu His Pro Ile Ala His Arg Asp Met Val Ile Ile Gln Leu 1745 1750 1755	5279
CCA AAG GAU UUC CCA CCG UUC CCA AUG CGC UUG AAA UUC GAA CAA CCA Pro Lys Asp Phe Pro Pro Phe Pro Met Arg Leu Lys Phe Glu Gln Pro 1760 1765 1770 1775	5327
UCA CGA GAU AUG CGA GUC UGC CUA GUA GGA GUC AAC UUC CAA CAG AAU Ser Arg Asp Met Arg Val Cys Leu Val Gly Val Asn Phe Gln Gln Asn 1780 1785 1790	5375

UAU AGC ACU UGC AUC GUA UCA GAA AGU AGU GUG ACA GCA CCA AAA GGA Tyr Ser Thr Cys Ile Val Ser Glu Ser Ser Val Thr Ala Pro Lys Gly 1795 1800 1805	5423
AAU GGA GAC UUU UGG AAA CAU UGG AUA UCA ACA GUC GAC GGU CAA UGU Asn Gly Asp Phe Trp Lys His Trp Ile Ser Thr Val Asp Gly Gln Cys 1810 1815 1820	5471
GGA CUA CCA UUG GUA GAU ACU AAG AGC AAA CAU AUU GUC GGA AUU CAU Gly Leu Pro Leu Val Asp Thr Lys Ser Lys His Ile Val Gly Ile His 1825 1830 1835	5519
AGU CUU GCA UCA ACA AGU GGA AAC ACU AAU UUC UUU GUC GCU GUG CCU Ser Leu Ala Ser Thr Ser Gly Asn Thr Asn Phe Phe Val Ala Val Pro 1840 1845 1850 1855	5567
GAG AAC UUU AAU GAA UAC AUC AAU GGA CUC GUG CAA GCA AAU AAA UGG Glu Asn Phe Asn Glu Tyr Ile Asn Gly Leu Val Gln Ala Asn Lys Trp 1860 1865 1870	5615
GAA AAA GGA UGG CAC UAU AAU CCG AAU CUC AUA UCC UGG UGU GGA CUA Glu Lys Gly Trp His Tyr Asn Pro Asn Leu Ile Ser Trp Cys Gly Leu 1875 1880 1885	5663
AAU UUA GUU GAU UCA GGC CCA AAA GGU UUG UUU AAA ACG UCA AAA UUG Asn Leu Val Asp Ser Ala Pro Lys Gly Leu Phe Lys Thr Ser Lys Leu 1890 1895 1900	5711
GUA GAA GAC UUG GAC GCG AGC GUU GAA GAG CAA UGC AAG AUC ACC GAA Val Glu Asp Leu Asp Ala Ser Val Glu Glu Gln Cys Lys Ile Thr Glu 1905 1910 1915	5759
ACA UGG CUC ACA GAG CAA UUA CAA GAU AAU UUA CAA GUG GUU GCG AAA Thr Trp Leu Thr Glu Gln Leu Gln Asp Asn Leu Gln Val Val Ala Lys 1920 1925 1930 1935	5807
UGU CCA GGC CAA CUA GUU ACC AAG CAU GUU GUU AAG GGU CAA UGC CCA Cys Pro Gly Gln Leu Val Thr Lys His Val Val Lys Gly Gln Cys Pro 1940 1945 1950	5855
CAC UUU CAA UUG UAC UUA UCA ACA CAU GAC GAU GCU AAA GAA UAC UUC His Phe Gln Leu Tyr Leu Ser Thr His Asp Asp Ala Lys Glu Tyr Phe 1955 1960 1965	5903
GCA CCC AUG CUU GGA AAA UAC GAC AAG AGU AGG CUU AAC AGA GCA GCU Ala Pro Met Leu Gly Lys Tyr Asp Lys Ser Arg Leu Asn Arg Ala Ala 1970 1975 1980	5951
UUU AUC AAA GAC AUA UCA AAA UAU GCA AAA CCA AUU UAC AUU GGA GAA Phe Ile Lys Asp Ile Ser Lys Tyr Ala Lys Pro Ile Tyr Ile Gly Glu 1985 1990 1995	5999
AUC GAG UAU GAU AUC UUU GAU AGA GCU GUA CAG CGG GUU GUC AAU AUU Ile Glu Tyr Asp Ile Phe Asp Arg Ala Val Gln Arg Val Val Asn Ile 2000 2005 2010 2015	6047

CUC AAA AAU GUU GGA AUG CAA CAA UGC GUU UAU GUC ACA GAU GAA GAA Leu Lys Asn Val Gly Met Gln Gln Cys Val Tyr Val Thr Asp Glu Glu 2020 2025 2030	6095
GAA AUU UUC AGA UCA CUU AAC CUG AAC GCA GCU GUC GGA GCA UUG UAU Glu Ile Phe Arg Ser Leu Asn Leu Asn Ala Ala Val Gly Ala Leu Tyr 2035 2040 2045	6143
ACA GGA AAG AAG AAA AAU UAC UUU GAA AAU UUU UCA AGC GAA GAC AAA Thr Gly Lys Lys Lys Asn Tyr Phe Glu Asn Phe Ser Ser Glu Asp Lys 2050 2055 2060	6191
GAA GAA AUC GUG AUG AGA UCC UGU GAA CGU AUU UAC AAU GGS CAA CUU Glu Glu Ile Val Met Arg Ser Cys Glu Arg Ile Tyr Asn Xaa Gln Leu 2065 2070 2075	6239
GGC GUA UGG AAU GGA UCG CUC AAA GCU GAG AUC AGA CCA AUA GAG AAA Gly Val Trp Asn Gly Ser Leu Lys Ala Glu Ile Arg Pro Ile Glu Lys 2080 2085 2090 2095	6287
ACC AUG CUG AAU AAG ACU CGA ACC UUC ACA GCG GCC CCA UUA GAA ACU Thr Met Leu Asn Lys Thr Arg Thr Phe Thr Ala Ala Pro Leu Glu Thr 2100 2105 2110	6335
UUG CUC GGA GGA AAA GUG UGC GUG GAU GAU UUU AAU AAU CAA UUC UAU Leu Leu Gly Gly Lys Val Cys Val Asp Asp Phe Asn Asn Gln Phe Tyr 2115 2120 2125	6383
UCA CAU CAU UUA GAA GGU CCA UGG ACU GUU GGG AUA ACA AAA UUC UAU Ser His His Leu Glu Gly Pro Trp Thr Val Gly Ile Thr Lys Phe Tyr 2130 2135 2140	6431
GGA GGU UGG AAU CGC UUA CUG GAG AAG UUA CCA GAA GGA UGG GUU UAC Gly Gly Trp Asn Arg Leu Leu Glu Lys Leu Pro Glu Gly Trp Val Tyr 2145 2150 2155	6479
UGC GAU GCU GAC GGG UCU CAA UUU GAU AGU UCG UUA ACA CCA UAU CUC Cys Asp Ala Asp Gly Ser Gln Phe Asp Ser Ser Leu Thr Pro Tyr Leu 2160 2165 2170 2175	6527
AUC AAU GCA GUA UUA AAU AUU CGA UUG CAA UUU AUG GAA GAU UGG GAU Ile Asn Ala Val Leu Asn Ile Arg Leu Gln Phe Met Glu Asp Trp Asp 2180 2185 2190	6575
AUA GGA GCG CAA AUG CUA AAG AAC CUG UAC ACU GAG AUU GUU UAC ACA Ile Gly Ala Gln Met Leu Lys Asn Leu Tyr Thr Glu Ile Val Tyr Thr 2195 2200 2205	6623
CCA AUC GCA ACG CCA GAC GGA UCA AUC GUG AAG AAA UUC AAA GGU AAC Pro Ile Ala Thr Pro Asp Gly Ser Ile Val Lys Lys Phe Lys Gly Asn 2210 2215 2220	6671
AAU AGC GGA CAA CCU UCU ACA GUA GUG GAC AAC ACA UUG AUG GUU AUA Asn Ser Gly Gln Pro Ser Thr Val Val Asp Asn Thr Leu Met Val Ile	6719

2225	2230	2235	
AUA GCU UUC AAC UAU GCC AUG CUA UCA AGU GGU AUC AAA GAA GAA GAA Ile Ala Phe Asn Tyr Ala Met Leu Ser Ser Gly Ile Lys Glu Glu Glu 2240	2245	2250	2255 6767
AUC GAU AAU UGC UGU AGA AUG UUC GCG AAU GGU GAU GAC UUA CUC CUA Ile Asp Asn Cys Cys Arg Met Phe Ala Asn Gly Asp Asp Leu Leu Leu 2260	2265	2270	6815
GCA GUG CAU CCU GAU UUU GAG UUC AUU UUA GAU GAA UUU CAA AAU CAC Ala Val His Pro Asp Phe Glu Phe Ile Leu Asp Glu Phe Gln Asn His 2275	2280	2285	6863
UUU GGG AAU CUU GGG CUG AAC UUC GAA UUU ACA UCA CGA ACA CGA GAU Phe Gly Asn Leu Gly Leu Asn Phe Glu Phe Thr Ser Arg Thr Arg Asp 2290	2295	2300	6911
AAA UCC GAA CUG UGG UUC AUG UCC ACA AGA GGC AUC AAG UAU GAA GGA Lys Ser Glu Leu Trp Phe Met Ser Thr Arg Gly Ile Lys Tyr Glu Gly 2305	2310	2315	6959
AUU UAC AUA CCA AAG CUU GAG AAA GAA AGA AUA GUC GCC AUA CUU GAA Ile Tyr Ile Pro Lys Leu Glu Lys Glu Arg Ile Val Ala Ile Leu Glu 2320	2325	2330	2335 7007
UGG GAU CGA UCA AAC UUG CCU GAA CAU AGG UUG GAA GCU AUA UGU GCA Trp Asp Arg Ser Asn Leu Pro Glu His Arg Leu Glu Ala Ile Cys Ala 2340	2345	2350	7055
GCG AUG GUU GAG GCC UGG GGA UAU UCC GAU CUC GUU CAU GAA AUA CGA Ala Met Val Glu Ala Trp Gly Tyr Ser Asp Leu Val His Glu Ile Arg 2355	2360	2365	7103
AAG UUC UAU GCG UGG CUU UUG GAA AUG CAA CCU UUU GCA AAU CUC GCA Lys Phe Tyr Ala Trp Leu Leu Glu Met Gln Pro Phe Ala Asn Leu Ala 2370	2375	2380	7151
AAA NAA GGG UUG GCC CCA UAC AUU GCC GAG ACA GCA CUC CGC AAU CUC Lys Xaa Gly Leu Ala Pro Tyr Ile Ala Glu Thr Ala Leu Arg Asn Leu 2385	2390	2395	7199
UAU CUU GGA ACG GGU AUC AAA GAG GAA GAA AUU GAA AAA UAU CUU AAA Tyr Leu Gly Thr Gly Ile Lys Glu Glu Glu Ile Glu Lys Tyr Leu Lys 2400	2405	2410	2415 7247
CAA UUC AUU AAG GAU CUU CCC GGA UAC AUA GAA GAU UAC AAU GAA GAU Gln Phe Ile Lys Asp Leu Pro Gly Tyr Ile Glu Asp Tyr Asn Glu Asp 2420	2425	2430	7295
GUA UUC CAU CAG UCG GGA ACU GUU GAU GCG GGU GCA CAA GGC GGC AGU Val Phe His Gln Ser Gly Thr Val Asp Ala Gly Ala Gln Gly Gly Ser 2435	2440	2445	7343
GGA AGC CAA GGG ACA ACA CCA CCA GCA ACA GGU AGU GGA GCA AAA CCA			7391

Gly Ser Gln Gly Thr Thr Pro Pro Ala Thr Gly Ser Gly Ala Lys Pro 2450 2455 2460	
GCC ACC UCA GGG GCA GGA UCU GGU AGU GAC ACA GGA GCU GGA ACU GGU Ala Thr Ser Gly Ala Gly Ser Gly Ser Asp Thr Gly Ala Gly Thr Gly 2465 2470 2475	7439
GUA ACU GGA AGU CAA GCA AGG ACU GGC AGU GGC ACU GGG ACG GGA UCU Val Thr Gly Ser Gln Ala Arg Thr Gly Ser Gly Thr Gly Thr Gly Ser 2480 2485 2490 2495	7487
GGA GCA ACC GGA GGC CAA UCA GGA UCU GGA AGU GGC ACU GAA CAG GUU Gly Ala Thr Gly Gly Gln Ser Gly Ser Gly Ser Gly Thr Glu Gln Val 2500 2505 2510	7535
AAC ACG GGU UCA GCA GGA ACU AAU GCA ACU GGA GGC CAA AGA GAU AGG Asn Thr Gly Ser Ala Gly Thr Asn Ala Thr Gly Gly Gln Arg Asp Arg 2515 2520 2525	7583
GAU GUG GAU GCA GGC UCA ACA GGA AAA AUU UCU GUA CCA AAG CUC AAG Asp Val Asp Ala Gly Ser Thr Gly Lys Ile Ser Val Pro Lys Leu Lys 2530 2535 2540	7631
GCC AUG UCA AAG AAA AUG CGC UUA CCU AAA GCA AAA GGA AAA GAU GUG Ala Met Ser Lys Lys Met Arg Leu Pro Lys Ala Lys Gly Lys Asp Val 2545 2550 2555	7679
CUA CAU UUG GAU UUU CUA UUG ACA UAC AAA CCA CAA CAA CAA GAC AUA Leu His Leu Asp Phe Leu Leu Thr Tyr Lys Pro Gln Gln Gln Asp Ile 2560 2565 2570 2575	7727
UCA AAC ACU AGA GCA ACC AAG GAA GAG UUU GAU AGA UGG UAU GAU GCC Ser Asn Thr Arg Ala Thr Lys Glu Glu Phe Asp Arg Trp Tyr Asp Ala 2580 2585 2590	7775
AUA AAG AAG GAA UAC GAA AUU GAU GAC ACA CAA AUG ACA GUU GUC AUG Ile Lys Lys Glu Tyr Glu Ile Asp Asp Thr Gln Met Thr Val Val Met 2595 2600 2605	7823
AGU GGC CUU AUG GUA UGG UGC AUC GAA AAU GGU UGC UCA CCA AAC AUA Ser Gly Leu Met Val Trp Cys Ile Glu Asn Gly Cys Ser Pro Asn Ile 2610 2615 2620	7871
AAC GGA AAU UGG ACA AUG AUG GAU AAA GAU GAA CAA AGG GUC UUC CCA Asn Gly Asn Trp Thr Met Met Asp Lys Asp Glu Gln Arg Val Phe Pro 2625 2630 2635	7919
CUC AAA CCG GUC AUU GAG AAU GCA UCU CCA ACU UUC CGA CAA AUU AUG Leu Lys Pro Val Ile Glu Asn Ala Ser Pro Thr Phe Arg Gln Ile Met 2640 2645 2650 2655	7967
CAU CAU UUC AGU GAU GCA GCU GAA GCG UAC AUA GAG UAC AGA AAC UCU His His Phe Ser Asp Ala Ala Glu Ala Tyr Ile Glu Tyr Arg Asn Ser 2660 2665 2670	8015

(2) INFORMATION FOR SEQ ID NO:2:

SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 2763 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Glu Glu Lys Gln Arg Glu Tyr Leu Ala Lys Asp Gln Lys Leu Ser Arg
1 5 10 15

Met Ile Gln Phe Ile Lys Glu Arg Cys Asn Pro Lys Phe Ser His Leu
20 25 30

Pro Thr Leu Trp Gln Val Ala Glu Thr Ile Gly is Tyr Thr Asp Asn
35 40 45

- 45 -

Gln Ser Lys Gln Ile Met Asp Val Ser Glu Ala Leu Ile Lys Val Asn
 50 55 60

Thr Leu Thr Pro Asp Asp Ala Met Lys Ala Ser Ala Ala Leu Leu Glu
 65 70 75 80

Val Ser Arg Trp Tyr Lys Asn Arg Lys Glu Ser Leu Lys Thr Asp Ser
 85 90 95

Leu Glu Ser Phe Arg Asn Lys Ile Ser Pro Lys Ser Thr Ile Asn Ala
 100 105 110

Ala Leu Met Cys Asp Asn Gln Leu Asp Lys Asn Ala Asn Phe Val Trp
 115 120 125

Gly Asn Arg Glu Tyr His Ala Lys Arg Phe Phe Ala Asn Tyr Phe Xaa
 130 135 140

Ala Val Asp Pro Thr Asp Ala Tyr Glu Lys His Val Thr Arg Phe Asn
 145 150 155 160

Pro Asn Gly Gln Arg Lys Leu Ser Ile Gly Lys Leu Val Ile Pro Leu
 165 170 175

Asp Phe Gln Lys Ile Arg Glu Ser Phe Val Gly Leu Ser Ile Asn Arg
 180 185 190

Gln Pro Leu Asp Lys Cys Cys Val Ser Lys Ile Glu Gly Gly Tyr Ile
 195 200 205

Tyr Pro Cys Cys Cys Val Thr Thr Glu Phe Gly Lys Pro Ala Tyr Ser
 210 215 220

Glu Ile Ile Pro Pro Thr Lys Gly His Ile Thr Ile Gly Asn Ser Ile
 225 230 235 240

Asp Ser Lys Ile Val Asp Leu Pro Asn Thr Thr Pro Pro Ser Met Tyr
 245 250 255

Ile Ala Lys Asp Gly Tyr Cys Tyr Ile Asn Ile Phe Leu Ala Ala Met
 260 265 270

Ile Asn Val Asn Glu Glu Ser Ala Lys Asp Tyr Thr Lys Phe Leu Arg
 275 280 285

Asp Glu Leu Val Glu Arg Leu Gly Lys Trp Pro Lys Leu Lys Asp Val
 290 295 300

Ala Thr Ala Cys Tyr Ala Leu Ser Val Met Phe Pro Glu Ile Lys Asn
 305 310 315 320

Ala Glu Leu Pro Pro Ile Leu Val Asp His Glu Asn Lys Ser Met His
 325 330 335

Val Ile Asp Ser Tyr Gly Ser Leu Ser Val Gly Phe His Ile Leu Lys

340	345	350
Ala Ser Thr Ile Gly Gln Leu Ile Lys Phe Gln Tyr Glu Ser Met Asp		
355	360	365
Ser Glu Met Arg Glu Tyr Ile Val Gly Gly Thr Leu Thr Gln Gln Thr		
370	375	380
Phe Asn Thr Leu Leu Lys Met Leu Thr Lys Asn Met Phe Lys Pro Glu		
385	390	395
Arg Ile Lys Gln Ile Ile Glu Glu Glu Pro Phe Leu Leu Met Met Ala		
405	410	415
Ile Ala Ser Pro Thr Val Leu Ile Ala Leu Tyr Asn Asn Cys Tyr Ile		
420	425	430
Glu Gln Ala Met Thr Tyr Trp Ile Val Lys Asn Gln Gly Val Ala Ala		
435	440	445
Ile Phe Ala Gln Leu Glu Ala Leu Ala Lys Lys Thr Ser Gln Ala Glu		
450	455	460
Leu Leu Val Leu Gln Met Gln Ile Leu Glu Lys Ala Ser Asn Gln Leu		
465	470	475
Arg Leu Ala Val Ser Gly Leu Ser His Ile Asp Pro Ala Lys Arg Leu		
485	490	495
Leu Trp Ser His Leu Glu Ala Met Ser Thr Arg Ser Glu Met Asn Lys		
500	505	510
Glu Leu Ile Ala Glu Gly Tyr Ala Leu Tyr Asp Glu Arg Leu Tyr Thr		
515	520	525
Leu Met Glu Lys Ser Tyr Val Asp Gln Leu Asn Gln Ser Trp Ala Glu		
530	535	540
Leu Ser Tyr Cys Gly Lys Phe Ser Ala Ile Trp Arg Val Phe Arg Val		
545	550	555
Arg Lys Tyr Tyr Lys Pro Ser Leu Thr Val Arg Lys Ser Val Asp Leu		
565	570	575
Gly Ala Val Tyr Asn Ile Ser Ala Thr His Leu Ile Ser Asp Leu Ala		
580	585	590
Arg Lys Ser Gln Asp Gln Val Ser Ser Ile Leu Thr Lys Leu Arg Asn		
595	600	605
Gly Phe Tyr Asp Lys Leu Glu Lys Val Arg Ile Arg Thr Ile Lys Thr		
610	615	620
Val Tyr Trp Phe Ile Pro Asp Ile Phe Arg Leu Val His Ile Phe Ile		
625	630	635
		640

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Val Leu Ser Leu Leu Thr Thr Ile Ala Asn Thr Ile Ile Val Thr Met
 645 650 655
 Asn Asp Tyr Lys Lys Leu Lys Lys Gln Gln Arg Glu Asp Glu Tyr Glu
 660 665 670
 Ala Glu Ile Ser Glu Val Arg Arg Ile His Ser Thr Leu Met Glu Glu
 675 680 685
 Arg Lys Asp Asn Leu Thr Cys Glu Gln Phe Ile Glu Tyr Met Arg Xaa
 690 695 700
 Asn His Pro Arg Leu Val Gly Xaa Thr Leu Asp Leu Thr His Thr Gly
 705 710 715 720
 Val Ile His Glu Gly Lys Ser Asn Leu Glu Thr Asn Leu Glu Gln Ser
 725 730 735
 Met Ala Val Gly Thr Leu Ile Thr Met Ile Leu Asp Pro Gln Lys Ser
 740 745 750
 Asp Ala Val Tyr Lys Val Leu Asn Lys Met Arg Thr Val Ile Ser Thr
 755 760 765
 Ile Glu Gln Asn Val Pro Phe Pro Ser Val Asn Phe Ser Asn Ile Leu
 770 775 780
 Thr Pro Pro Val Ala Gln Gln Ser Val Asp Val Asp Glu Pro Leu Thr
 785 790 795 800
 Leu Ser Thr Asp Lys Asn Leu Thr Ile Asp Phe Asp Thr Asn Gln Asp
 805 810 815
 Leu Pro Ala Asp Thr Phe Ser Asn Asp Val Thr Phe Xaa Asp Trp Trp
 820 825 830
 Ser Xaa Gln Leu Ser Asn Asn Arg Thr Val Xaa His Tyr Arg Xaa Trp
 835 840 845
 Gly Glu Ser Xaa Leu Glu Phe Thr Arg Glu Asn Ala Ala His Thr Ser
 850 855 860
 Ile Glu Leu Ala His Ser Asn Ile Glu Arg Glu Phe Leu Leu Arg Gly
 865 870 875 880
 Ala Val Gly Ser Gly Lys Ser Thr Gly Leu Pro Tyr His Leu Ser Met
 885 890 895
 Arg Gly Lys Val Leu Leu Leu Glu Pro Thr Arg Pro Leu Ala Glu Asn
 900 905 910
 Val Cys Arg Gln Leu Gln Gly Pro Pro Phe Asn Val Ser Pro Thr Leu
 915 920 925

Gln Met Arg Gly Leu Ser Ser Phe Gly Cys Thr Pro Ile Thr Ile Met
 930 935 940
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 1155 1160 1165
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 1170 1175 1180
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 1185 1190 1195 1200
 Leu Ala Lys Cys Thr Val Lys Gln Ala Arg Thr Met Met Gln Phe Glu
 1205 1210 1215
 Leu Ser Pro Phe Val Met Ala Glu Leu Val Lys Phe Asp Gly Ser Met

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- 54 -

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Thr Gln Glu Asn Thr Glu Arg His Thr Ala Gly Asp Val Ser Arg Asn
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Met His Ser Leu Leu Gly Val Gln Gln His His
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(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 20 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

- (A) DESCRIPTION: /desc = "first Adh internal control primer"

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

TGCATGTCGG TTGTGTTGCA

20

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 20 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

- (A) DESCRIPTION: /desc = "second Adh internal control primer"

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

20

CTCAGCAAGT ACCTAGACCA

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 19 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

- (A) DESCRIPTION: /desc = "first synthetic PAT gene primer"

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

TGTCTCCGGA GAGGAGACC

19

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 20 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

- (A) DESCRIPTION: /desc = "second synthetic PAT gene primer"

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

CCAACATCAT GCCATCCACC

20

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 33 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

- (A) DESCRIPTION: /desc = "first N1a proteinase gene primer"

- 56 -

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

GCGGGATCCA TGGGGAAGAA CAAACGCAGT TGA

33

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 30 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(A) DESCRIPTION: /desc = "second NIa proteinase
primer"

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

GCGGAGCTCT TACTCTTCAA CGCTCGCGTC

30

INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 13bis)

A. The indications made below relate to the microorganism referred to in the description on page <u>10</u> , line <u>34</u>	
B. IDENTIFICATION OF DEPOSIT Further deposits are identified on an additional sheet <input type="checkbox"/>	
Name of depositary institution	Agricultural Research Service Culture Collection (NRRL)
Address of depositary institution (including postal code and country) 1815 North University Street Peoria, IL 61604 USA	
Date of deposit 29 June 1995 (29.06.95)	Accession Number NRRL B-21479
C. ADDITIONAL INDICATIONS (leave blank if not applicable) This information is continued on an additional sheet <input type="checkbox"/>	
We request the Expert Solution where available	
D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE (if the indications are not for all designated States)	
E. SEPARATE FURNISHING OF INDICATIONS (leave blank if not applicable)	
The indications listed below will be submitted to the International Bureau later (specify the general nature of the indications e.g., "Accession Number of Deposit")	
<div style="display: flex; justify-content: space-between;"><div style="width: 45%;"><p>For receiving Office use only</p><p><input checked="" type="checkbox"/> This sheet was received with the international application</p><p>Authorized officer <i>R.L.R. Pether</i> R.L.R. Pether</p></div><div style="width: 45%;"><p>For International Bureau use only</p><p><input type="checkbox"/> This sheet was received by the International Bureau on:</p><p>Authorized officer</p></div></div>	

What is claimed is:

1. A chimeric gene comprising a monocotyledonous plant promoter operably linked to a nucleotide sequence derived from the genomic sequence of a virus infecting monocotyledonous plants, wherein said nucleotide sequence contains a modification rendering a messenger RNA transcribed from said nucleotide sequence incapable of complete translation.
2. The chimeric gene of claim 1 wherein said virus is selected from the group consisting of a potyvirus, a luteovirus, a tenuivirus, a carmovirus, a machlovirus, a geminivirus and a reovirus.
3. The chimeric gene of claim 2 wherein said virus is a potyvirus.
4. A chimeric gene comprising a monocotyledonous plant promoter operably linked to a nucleotide sequence derived from the genomic sequence of a maize dwarf mosaic virus, wherein said nucleotide sequence contains a modification rendering a messenger RNA transcribed from said nucleotide sequence incapable of complete translation.
5. The chimeric gene of claim 4 wherein said virus is maize dwarf mosaic virus strain B.
6. The chimeric gene of claim 4 wherein said transcribed RNA is capable of translating an attenuated peptide of a maize dwarf mosaic virus protein.
7. The chimeric gene of claim 6 wherein said attenuated peptide is less than 20 amino acids in length.
8. The chimeric gene of claim 4 wherein said transcribed RNA cannot be translated.
9. The chimeric gene of claim 4 wherein said transcribed RNA sequence does not include the translation initiation codon of said maize dwarf mosaic virus, strain B.
10. The chimeric gene of claim 4 wherein said transcribed RNA sequence encodes a portion of a viral protein selected from the group consisting of a coat protein, a proteinase, a replicase, a helicase, a Vpg protein, a 6K protein and a helper component.

11. The chimeric gene of claim 4 wherein said modification comprises addition of a premature stop codon into said transcribed RNA.
12. The chimeric gene of claim 4 wherein expression of said gene in transgenic maize, sorghum or sugarcane inhibits infection of said transgenic plants by maize dwarf mosaic virus.
13. The chimeric gene of claim 12 wherein expression of said gene in transgenic maize inhibits infection of the transgenic plants by maize dwarf mosaic virus.
14. The chimeric gene of claim 5 wherein said transcribed RNA comprises nucleotides 4452 to 5744 of SEQ ID No. 1 and said modification comprises the substitution of a T for the A at position 4470 of SEQ ID No. 1.
15. The chimeric gene of claim 14 wherein said modification further comprises the insertion of an ATG codon immediately before the G at position 4452 of SEQ ID No. 1.
16. The chimeric gene of claim 4 wherein said monocotyledonous plant promoter is selected from the group consisting of a maize ubiquitin promoter, a maize actin promoter and a maize phosphoenolpyruvate carboxylase promoter.
17. A method for producing a monocotyledonous plant with an inheritable trait of resistance to infection by a maize dwarf mosaic virus comprising transforming said plant with a chimeric gene according to claim 4.
18. A monocotyledonous plant having an inheritable trait of resistance to infection by a maize dwarf mosaic virus, wherein said plant comprises a chimeric gene according to claim 4.
19. A chimeric gene comprising a plant promoter operably linked to a nucleotide sequence derived from the genomic sequence of maize dwarf mosaic virus strain B encoding a viral protein other than a coat protein, wherein transgenic expression of said chimeric gene in a plant inhibits infection of said plant with said virus.
20. The chimeric gene according to claim 19 wherein said viral protein is selected from the group consisting of RNA dependent RNA polymerase (RdRp) having the amino acid sequence from position 1915 to 2435 of SEQ ID No. 2, Nla proteinase having the

amino acid sequence from position 1484 to 1914 of SEQ ID No. 2, helicase having the amino acid sequence from position 792 to 1430 of SEQ ID No. 2, and P3 proteinase having the amino acid sequence from position 378 to 791 of SEQ ID No. 2.

21. The chimeric gene of claim 20 wherein said viral protein is a replicase.
22. The chimeric gene of claim 20 wherein said plant promoter is selected from the group consisting of a plant ubiquitin gene promoter, a plant actin gene promoter, and a plant pith-preferred promoter.
23. A method for producing a plant with an inheritable trait of resistance to infection by maize dwarf mosaic virus strain B comprising transforming said plant with the chimeric gene of claim 19.
24. A plant comprising the chimeric gene of claim 22.
25. A method for protecting progeny of a monocotyledoneous parent plant from viral infection comprising transforming said parent plant with a chimeric gene according to claim 1 and obtaining progeny plants or breeding said parent plant with a plant according to claim 18.
26. A method according to claim 25, wherein said progeny are protected from infection with maize dwarf mosaic virus.
27. A method according to claim 25, wherein the progeny of maize, sorghum or sugarcane plants are protected from viral infection.

INTERNATIONAL SEARCH REPORT

International Application No
PCT/EP 96/02673

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/82 C12N15/40 A01N63/02 A01H5/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP,A,0 578 627 (MONSANTO CO) 12 January 1994	19,23
Y	see the whole document	10
Y	WO,A,93 14210 (SANDOZ AG ;SANDOZ AG (DE); SANDOZ LTD (CH)) 22 July 1993	1-9, 11-13, 17,18, 25-27
	see the whole document	
Y	WO,A,93 17098 (OREGON STATE) 2 September 1993	1-13,17, 18,25-27
	see the whole document	

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☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

* Special categories of cited documents:

- *A* document defining the general state of the art which is not considered to be of particular relevance
- *E* earlier document but published on or after the international filing date
- *L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- *O* document referring to an oral disclosure, use, exhibition or other means
- *P* document published prior to the international filing date but later than the priority date claimed

- *T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- *X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- *Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
- *&* document member of the same patent family

Date of the actual completion of the international search

4 December 1996

Date of mailing of the international search report

11. 12. 96

Name and mailing address of the ISA
European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl,
Fax (+ 31-70) 340-3016

Authorized officer

Holtorf, S

INTERNATIONAL SEARCH REPORT

Inter nal Application No
PCT/EP 96/02673

C(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	THE PLANT CELL, vol. 6, October 1994, pages 1329-1331, XP002019958 CHASAN, R.: "MAKING SENSE (SUPPRESSION) OF VIRAL RNA-MEDIATED RESISTANCE" see the whole document ---	1-27
A	THE PLANT CELL, vol. 6, October 1994, pages 1441-1453, XP002019959 SMITH, H.A., ET AL.: "TRANSGENIC PLANT VIRUS RESISTANCE MEDIATED BY UNTRANSLATABLE SENSE RNAs: EXPRESSION, REGULATION, AND FATE OF NONESSENTIAL RNAs" see the whole document ---	1-27
A	WO,A,95 04825 (UNILEVER PLC ;UNILEVER NV (NL); STRATFORD REBECCA (GB); BOULTON RO) 16 February 1995 see the whole document ---	10,21
A	EP,A,0 342 926 (LUBRIZOL GENETICS INC) 23 November 1989 see the whole document -----	16,22

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/EP 96/02673

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WO-A-9314210	22-07-93	US-A- 5530193	25-06-96
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WO-A-9317098	02-09-93	CA-A- 2130454 EP-A- 0626998 JP-T- 8500003 NO-A- 943038	02-09-93 07-12-94 09-01-96 17-10-94
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WO-A-9504825	16-02-95	AU-A- 1318395	28-02-95
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EP-A-0342926	23-11-89	AT-T- 112314 DE-D- 68918494 DE-T- 68918494 ES-T- 2060765 JP-A- 2079983 US-A- 5510474	15-10-94 03-11-94 23-03-95 01-12-94 20-03-90 23-04-96
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